TOLERANCES OF CERTAIN CITRUS SEEDLINGS TO FREE WATER IN SOIL

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

Citrus is being planted on poorly drained flatwoods soil in Florida because of the increasing demand for more citrus land. Observations rather than experimental data have been reported on the water tolerance of citrus plants used as rootstocks. Experimental data for the tolerance of different rootstocks in relation to a specific soil type would provide basic information that should be of value in determining the suitability of a site for citrus.

This study was made to ascertain under laboratory and greenhouse conditions the length of time Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings will tolerate free water in Leon fine sand, a flatwoods soil type of vast acreage in Florida on which plantings of citrus are being made.

In addition to the investigations of the water tolerance of the different citrus seedlings, experiments were made to test the hypothesis that toxic substances cause injury to the seedlings when the soil in which the seedlings are grown is flooded. A study was also made of the possibility of determining when a root is dead in advance of injury to the top of the plant.

II. REVIEW OF LITERATURE

Flood Injury

Saturating the soil with water causes injury to or death of many species of plants. The injury or death of the root system has been attributed to deficient aeration accompanying flooding. This does not explain why the shoots are injured rather quickly in some species of plants and more slowly in others. Also, this does not account for the type of injury which occurs in all susceptible plants. The injury of plants in such saturated soils is usually attributed to desiccation, caused by decreased water absorption through the injured roots. Kramer (50) did not consider this an adequate explanation. Aerial portions of plants have been shown to live for some days after the root systems were killed if the soil was kept saturated (47) or if the roots were placed in fresh water (84). The injury of shoots cannot be caused entirely by injury to the roots as absorbing systems, because reduced absorption of water or of minerals cannot explain all of the symptoms observed in the shoots of flooded plants (50). Wilting of leaves is often observed after flooding but this is not the only or even the most characteristic symptom of injury.

Among the conspicuous symptoms of flooding injury is yellowing and death of the leaves, beginning with the lower ones and progressing up the stem (2,38,50). This chlorosis superficially resembles nitrogen deficiency but often develops within four to six days after flooding, much too soon to be caused by nitrogen deficiency. The middle leaves of tomato showed epinastic curvature within twenty-four to forty-eight hours after the soil was flooded. This epinasty was almost as severe on tomato plants which were in circulating tap water as on plants in oxygen-free water (38). Jackson (38) points out that epinasty is induced by a slight oxygen deficiency and, presumably, by light injury to the roots. Lumps of callus tissue develop along the stem, particularly at the water level or in many species at the soil surface or just below the water surface where the water level is above the soil surface. Jackson (37) also found that adventitious roots did not prevent injury to shoots of Marglobe tomato plants when the original roots were flooded, but leaf epinasty was less and shoot growth was greater than the flooded plants without adventitious roots.

Except for aquatically adapted species, plants which are growing in soil saturated with water soon have injured root systems which cause the leaves to yellow, reduce growth and eventually die (50). This

injury of the root systems has been attributed to the lack of oxygen and possibly to the accumulation of carbon dioxide, rather than to the direct effects of water. The reason most often given to support this gaseous concept of injury is that most species of plants make satisfactory growth in well-aerated water cultures.

Attempts (9,28) have been made to measure the oxygen and carbon dioxide content in the gaseous phase of the soil while saturated with water and as the soil drained. Scott and Evans (80) point out that a measurement of the dissolved gases in the liquid phase of the soil should be of considerable value in characterizing the aeration of a soil. Furr (27) concluded there was no relation of the root rot of citrus and avocado to low oxygen or high carbon dioxide under field conditions. Respiration of soil organisms and of roots continually depletes the oxygen and adds to the carbon dioxide of the soil atmosphere and of the water film in equilibrium with it. The activity of soil organisms varies with temperature, moisture, and supply of organic matter that they can use as food.

Karsten (40) points out that if the oxygen content of the soil is reduced to such an extent that the rate of basal respiration is radically lowered, the roots of the

plant will die and death of the tops will ensue. If the reduction is not sufficient to cause death it is sufficient to impair the growth of the roots, and this condition will be reflected in reduced growth and reduced productivity of the aerial portion of the plants. Floyd (24) made no gaseous measurements in his experiment, but the root system of the citrus plants was impaired by the water level in the soil and the tops of the citrus trees reflected this injury. Conway (17) points out that indirect effects of an oxygen deficit are important to keep in mind when investigating the supply of oxygen to aquatics, besides the measurements of oxygen in the soil atmosphere or in solution. Redox potentials may be of importance in root respiration apart from their use as indicating oxygen concentrations. Black (4) proposed four different hypotheses that might account for the failure in measuring the composition of soil air which reflects the apparent aeration condition of the soil: (1) technique used; (2) the variation between the oxygen and carbon dioxide concentration near the roots and that in the bulk sample; (3) the difference in the diffusion of dissolved gases through the surrounding water film; (4) when the oxygen concentration in the soil air decreases there may be an associated increase in certain unfavorable effects that do not occur in solution culture

experiments. The rate of transpiration, the oxygen supply and the temperature are cloeely related to the injury of plants. Heinicke (34) found that flooding the soil containing apple roots during the winter caueed coneiderable loee of small roote but produced no eerioue injury if the soil was drained before leaves began to appear. Flooding in the summer soon caused injury, particularly if the transpiration was rapid. Kramer (48) found that when the oxygen content wae lowered and the carbon dioxide increaeed by the addition of it there wae a marked reduction in the tranepiration of the tomato plante. However, when the oxygen was removed with oxygen-free nitrogen there was only a small decreaee in the water intake. The nitrogen gas would remove not only the oxygen eurrounding the roots, but aleo the carbon dioxide which otherwise might accumulate and retard respiration and active abeorption. Kramer also pointed out that if a high concentration of carbon dioxide or low concentration of oxygen wae maintained for many houre or daye, other factore became important. Root growth wae usually stopped (12,16, 31,57) and many or all of the roote might be killed, resulting in a greatly decreased abeorbing eurface. There ie also the poeeibility of injury to the shoot by toxic substances escaping from the dead cells, and plugging of the water conducting elemente may occur. One or more of these

factors may cause the death of plants with roots in poorly aerated soils. The specific effects of carbon dioxide on permeability of the roots and henos on water intake are probably most important during the early part of a period of poor aeration such as occurs in waterlogged soils. Both Chang and Loomis (13) and Kramer (48) concluded that the effect of carbon dioxide seemed to be on the water-absorption mechanism rather than on transpiration. Another effect of carbon dioxide was on the protoplasm and caused an increase in viscosity and a decrease in permeability (26,81).

The theory that oxygen made available by nitrate reduction would be beneficial to plants under waterlogged conditions was investigated by Bain and Chapman (2). Heavy applications of nitrates aggravated the waterlogged injury to avocados and grapefruit plants. All of the waterlogged grapefruit plants began to develop vein-chlorotic leaves after about ten days and the severity increased with time. This condition developed when serious root rotting occurred. There was no significant difference between nitrate-treated and non-nitrate-treated plants. Klotz and Sokoloff (44) reported that flooded sour orange and sweet orange seedlings which had received nitrate only and those which had no organic matter or nitrate added were not wilted after four weeks of flooding;

whereas those seedlings that were given organic matter and nitrate nitrogen were in a state of collapse. They showed that initial injury to the roots could occur several months prior to the appearance of collapse. The toxic substance, nitrite, may disappear from the root zone long before injury to roots or to the leaves may become visible. In water cultures the toxic nitrite ion very rapidly injures the roots, initially increases and later decreases their respiration, makes them more permeable, and permits exosmosis of materials from them. In order for nitrites to accumulate in soil it has been proposed (14,82) that certain conditions must be met: the pH of soil must be above 7.0 for nitrate-reducing bacteria, a high proportion of organic matter must be available for the reduction of nitrate, the soil must be well aerated, and the soil temperature must be favorable. Chapman and Liebig (14) concluded that the reduction reaction was more likely to take place in deeper layers, where there was a static water table or impeded drainage which led to saturated and anaerobic conditions, than in surface layers. Zentmeyer and Bingham (93) could not demonstrate that the nitrite-nitrogen accelerated root rot by Phytophthora nor that root injury caused by nitrite increased the rapidity or severity of root attack by the fungus. The growth of sweet orange

seedlings was retarded because of the amount of root rot caused by Phytophthora spp. (45). Environmental factors favoring the parasitism of the fungi are excess water and organic matter in the soil.

Soil Toxins

Soil toxins are probably related to deficient aeration and to anaerobic conditions (79). Clements (16) stated that this could be shown by the fact that they were readily oxidized and soon disappeared under proper tillage. The toxins appeared to be due to essentially the same conditions and processes as obtained in bogs. Livingston (56) and Dachnowski (19) showed that bog water contained toxic substances and that these substances were stable to boiling for ten minutes (56) but could be removed by shaking with carbon black or calcium carbonate (19). The toxicity of these waters is not due to acidity nor to lack of oxygen (19). In contrast Clements (16) states that the primary causes of toxicity are the direct lack of oxygen and its indirect effect in permitting the accumulation of carbon dioxide in harmful amounts which stimulate the production of injurious organic acids and other compounds. In many cases probably the first two alone are concerned, but in sour soils and muck soils all of them must have a part though the lack of oxygen plays the primary role.

Clements (16) concluded that organic toxins are excreted by roots or produced in soils only as a consequence of the anaerobic respiration of plant roots and of microorganisms, and that the inorganic toxins may arise as a result of chemical processes of adsorption. Russell (79) concluded that toxins may occur on sour soils poorly aerated and lacking in calcium carbonate, or in other exhausted soils, whereas there is no evidence of soluble toxins in normally aerated soils sufficiently supplied with mineral nutrients and with calcium carbonate.

Finely divided material has a marked inhibitory action on the toxicity of many solutions (88). The beneficial effect has generally been ascribed to the physical phenomenon of adsorption. In soils there are large surface exposures and adsorption may play a large part in inhibiting the action of plant toxins. The great complexity of soil constituents suggests the possibility that plant toxins may combine chemically with certain soil constituents and thus be removed at least partly from the soil solution, resulting in a greatly lessened toxic action to plants. Chemical reactions are probably important in lessening the harmful effects of plant toxins in soils. For example, calcium carbonate inhibits the toxicity of copper salts and kaolin or an acid clay soil inhibits the toxic effects of the

strong base guanidine. The latter inhibition is attributed to the reaction of the acid nature and base. Undoubtedly the chemical reactions play fully as important a role as physical phenomena such as adsorption and possibly the former have the greater effect.

Inorganic constituents can be toxic to plants. Robinson (75) gives evidence that submerged soils contain abnormally high concentrations of manganous and ferrous ions which render the soil solution toxic to most species. The substances are kept in solution as bicarbonates because of the high concentrations of carbon dioxide which result from the submergence of soils, and the presence of ferrous ions is a symptom of highly reducing conditions. Sulfides are produced in submerged soils and are very poisonous to plants, even in low concentrations.

In studies on the biochemistry of waterlogged soils (85) there was a distinct increase in the free and saline ammonia content and this was present mostly in the soil sediment. There was no release of any soluble reducing matter capable of absorbing dissolved oxygen nor was there any appreciable production of carbon dioxide. The fluctuations of dissolved oxygen in the waterlogged soil were attributed to variations

in external conditions with time and not to any soil factor. Waterlogging resulted in an increase in alkalinity which was associated with the corresponding increase in ammonia. Robinson (75) did not find that submergence affected the pH values very markedly; if anything, the pH fell slightly, which he attributed to the higher carbon dioxide concentration.

Substances have been extracted from cultivated soils which have proven to be growth inhibitors for succeeding crops of the same plants. It was found that toxic substances from the culture media of guayule inhibited growth of guayule under certain limited environmental conditions (7). These toxic agents, although they were not isolated in pure form, were characterized chemically as being ether-soluble acidic compounds. Cinnamic acid was one of the toxic agents isolated from water in which guayule roots had been briefly allowed to steep and is a normal constituent of the guayule plant. There was a considerable reduction in growth in both height and dry weight of guayule plants when one gram of cinnamic acid had been added per pot filled with 1500 grams of Hanford sandy loam (8). When leaves of Encelia farinosa were applied to tomato and other plants in sand cultures a striking growth inhibition occurred (33). Water and ether extract of the Encelia

leaves when supplied to tomato seedlings in solution culture may cause death of the plants within one day.

Weekly watering of fresh sand oultures of orange seedlings with the leachate from old sand cultures reduced growth by approximately one-third (60). When this sand previously cropped to citrus was leached with sulfuric acid followed by distilled water it produced growth comparable to that in the fresh sand. After sour or sweet orange seedlings had been grown in a medium quartz sand for eighteen months in the greenhouse the growth of a second crop of sweet orange seedlings in this sand was greatly retarded. In addition to detrimental organisms an organic toxic material apparently builds up gradually in soils cropped to citrus plants and is not readily leached from a normal soil by water but may be partly removed from a very sandy soil by leaching. This hypothetical toxic material could originate by slow excretion from the citrus roots or could be produced by microorganisms growing on root surfaces or dead root material. Because of the gradual build up and persistence in citrus soils as reflected by the reduced plant growth, toxic material was probably resistant to decay by soil organisms. The acid leaching suggests that it was either soluble in or destroyed by sufficiently strong acid. This toxin was found to be

specific to citrus in its toxic effect and not injurious to tomatoes or avocados. Wander (89) found that a methyl alcohol extract of grove soil when placed on virgin soil after the alcohol was removed caused the growth of grapefruit seedlings to be depressed as compared to seedlings grown in untreated soil. Ignition of the soil treated with methyl alcohol extract destroyed most of the inhibiting effect of the substance as reflected by the resultant growth of seedlings in the soil after ignition. A toxic material was extracted from the roots and leaves of diseased citrus trees that caused wilting of citrus and tomato cuttings in twenty-four hours after they were placed in the solution (86). This solution was made by covering 200 grams of wood from trees showing decline with water in a beaker and allowing it to steep for twenty-four hours at 45° F.

When peach roots were added to virgin soil the growth of peach seedlings was inhibited (70). In sand cultures the bark, but not the wood, of the roots was found to be toxic. The alcohol extract of bark also was toxic to peach seedlings. The injury was more severe to the root systems of the peach than to the tops when root bark was added. When leaching of the soil was slow, as when peat moss was present, the injury was greatest. Microbial decomposition of peach root residues produced a

toxic substance which is believed to be a factor involved in the difficult re-establishment of peach trees in old peach orchards (67).

Dried roots of brome grass were inhibitory to the growth of the same species (3). Benedict (3) suggested that the thinning out of brome grass stands may be the result of the accumulation of a toxic substance from the roots. It was also suggested that its living roots may also excrete substances toxic to the plant.

Rovira (76) showed that the roots of pea and oat plants when grown under aseptic conditions excreted amino acids, fructose, glucose and compounds which absorb and fluoresce ultraviolet. Katznelson et al. (43) were able to recover significant amounts of amino-nitrogen from the leachates of sand which had been dried until the wheat plants had begun to wilt and then the sand remoistened. Also detectable reducing compounds were liberated in these dried and remoistened pots. More total amino-nitrogen was found in the leachate from pots with tomato, soybean, barley and oats which were allowed to wilt then remoistened and leached than was found in leached water from the pots which were kept wet (42). They believe that in field soil, subjected to frequent drying and moistening, this phenomenon also occurs, thus providing the rhizosphere microflora with

a food supply and eepecially with amino-nitrogen. Anaerobic bacteria in general were coneietently etimulated in the rhizosphere of plante in both fertilized and unfertilized soil and were alwaye present in greater numbers on the roots in the latter (41). The treatment of soil with the solution of root exudate from pea and oat plants reeulted in increaeed numbers of gram-negative bacteria (78). Fungal counte in the treated soil ehowed no etimulation by the root exudate, indicating an action similar to that in the root environment in which bacteria are etimulated to a greater extent than fungi.

Many reeearchers have worked with toxins which are related to wilting in plants. Some have been concerned with the phyeiology of toxin formation in microorganisme (23,36), the production of toxic material by epecific organieme (10,15,21,29,35,69,87), defenee reaction of plants to the preeence of toxins (6), and with the baeie for toxic wilting (29). Gadmann (29) points out the difference between physiological wilting and toxic wilting. The former is caueed by a lack of water, therefore reversible. The toxic wilting ie caused by a colloid-chemical disturbance of the osmotic mechanism through the destruction of the oemotic prerequieites for turgor and because of this, toxic wilting

is irreversible. Wilting plants removed from a toxin solution to water or a nutrient solution continue to wilt and do not recover. The toxin exerts a coagulating effect on the plasma which leads to two pathological phenomena: (1) damage to the water-retaining capacity of the plasma resulting in pathological water loss; (2) damage to the semi-permeability of the plasma membrane which leads to a loss of turgor.

The injury, whether it is chlorosis or wilting, and death of leaves may be caused at least in part by toxic substances moving up from the dead roots or even from the solution in the surrounding soil (51). It is still unknown whether the injury is physiological or pathological or a combination. Many factors enter into the injury of shoots of flooded plants which make it complex in origin.

Detection of Viable Tissues

Reasons believed to cause plants to become injured in waterlogged and poorly aerated soils have been reviewed. The next question to be encountered is whether the injured tissues of the root system can be detected before visible symptoms appear on the top of the plant. Roots in advanced stages of injury are soft and spongy, and the cortical portion slips rather easily.

McPherson (63) immersed thick longitudinal sections of roots for one hour in various concentrations of different chemicals, then removed and tested them for the presence or absence of living cortical cells. Of the three vital stains tried-- congo red, methylene blue and neutral red-- the neutral red was the most satisfactory. The dead cells in the epidermis of Allium cepa took an intense orange color while the living cells became a cerise red color. In the cortical cells of corn roots, the living cells took on a bright red hue while the dead cells were colorless. The three criteria used to distinguish the living from the dead cells were vital staining, streaming and plasmolysis. The living cells stained, and in many cases streaming could be seen in the cytoplasm and plasmolysis took place readily when they were placed in a strong sucrose solution.

It was shown by Kuhn and Jerchel (52) that dilute solutions of 5-methyl and 5-hendecyl 2,3 diphenyl salts, as well as the 2,3,5-triphenyltetrazolium chloride, were capable of staining certain living cells such as bacteria, yeasts and garden cress. Such staining was brought about by a physiological reduction of the colorless triphenyltetrazolium salt to form the highly colored and insoluble triphenyl formazan.

Tetrazolium differs from the majority of physiological indicators (52) since, in the reduced state, it forms an insoluble formazan and the reaction is therefore non-reversible. This is advantageous in plant tissues. It is easily visible in minute quantities and the reaction is very sensitive. It was also possible to test the penetration of the indicator in plant tissues by reducing the tetrazolium with sodium hydrosulfite. Tetrazolium readily penetrates the majority of plant tissues and it is not absorbed. The formazan is insoluble and is neither diffused from the cell in which it was formed nor oxidized back to a colorless state on standing.

Tetrazolium has been used to predict the germinability of seeds (54). The seeds which germinated after soaking in a dilute solution of tetrazolium for several hours had deep red embryos.

Waugh (90) used a 1 per cent aqueous solution of tetrazolium to test the difference in response of tips of twigs, both heated in a test tube suspended in a boiling bath for fifteen minutes and unheated. All of the unheated sections which responded to the tetrazolium treatment developed a red coloration in the cambium layer. The heated sections of all varieties tested exhibited no coloration. The formazan appeared first in the cambium

but it took about four hours for the development of the red color. Exceptions to the length of time for the development of the red color were willow sections and rose cuttings. The cambium of the willow sections stained within 1-2 minutes, followed by slow development of color throughout the phloem. Rose cuttings required nearly twenty-four hours for the tetrazolium reduction.

Roberts (73) conducted a survey of tissues which reduce tetrazolium in vascular plants. Observations were made on the reduction zones in stem tissues of vascular plants. Reduction zones in root tissue were also observed. Actively growing root tips of all the plants surveyed showed some degree of reducing activity. Inner and outer cortical reduction regions were observed in meristematic root tip tissue. Plants possessing a tetrarch root system reduced tetrazolium in a tetrapolar pattern which corresponds to the pattern of secondary root formation.

Dufrenoy and Pratt (22) immersed freshly cut basal surfaces of culms of sugar cane in a 0.5 per cent aqueous solution of tetrazolium. The uncolored salt was rapidly transported to the upper nods and reduced there to the insoluble red formazan. Microscopic examination of longitudinal freehand sections showed that the precipitates of formazan were localized at the sites of the

plasmodesmata and in lipidic parts of the cytoplasm.

Lakon (54) states that the staining of embryos must be completed within twenty-four hours as micro-organisms will appear and obscure the reaction. Bacteria, stained by tetrazolium, may stimulate staining of cereal embryos or the cut surface of maize kernels. In this case the tetrazolium solution itself is stained red.

Apparently tetrazolium is reduced by several reducing substances. Jensen et al. (39) found that several dehydrogenase enzyme systems, prepared from corn embryos, in the presence of diphosphopyridine nucleotide were able to reduce tetrazolium. The presence of succinic dehydrogenase in tissue homogenates reduced the tetrazolium (53). This formazan was easily dissolved in acetone for colorimetric measurement. Reducing sugars in an alkaline medium are capable of reducing tetrazolium and the quantity of formazan is proportional to the quantity of reducing sugar present (62). The tetrazolium solution is reduced immediately on contact with a reductase system, hence the time of staining depends on the rate of diffusion of the solution (18). The reduction of tetrazolium could be inhibited by many compounds (26). From the lack of specificity for inhibitors it would seem that a number of reducing

enzymes acting on material inside the cell can reduce the dye. Aeration by shaking retarded the reduction, possibly because it raised the redox potential too high (over -0.08 volt) or because oxygen competed with the indicator. Tetrazolium can be reduced to the red formazan by ultraviolet light, alpha rays and x-rays (30). The formazan in visible light (480 mμ) turns from red to yellow. If the solution was subsequently placed in the dark, the formazan turned from yellow to red. This reversible conversion was caused by cis-trans isomerism. Roberts (74) and Brown (11) concur that the marked sensitivity of the reduction reaction to both temperature and light may well be due to an effect of the active sulfhydryl groups of the reducing enzymes.

Brown (11) found that split root tips or embryos exposed one-half to one hour in a 0.5-1.0 per cent aqueous solution of tetrazolium at room temperature and bright diffuse laboratory light were well stained. The root tips must be cut to stain quickly. The epidermis and outer layers of the cortex apparently prevent the rapid penetration of tetrazolium. The cells of these layers do not stain well even when the tip is split.

It was noted (11) in higher plants that

tetrazolium reduction occurred most obviously and quickly in meristematic cells. In the time required to stain the root tips older cells of the root were not stained at all. This could be due in part to the higher concentration of reducing enzyme in mitotic cells but certainly older cells must also contain these enzymes. Much longer treatment does produce staining of older cells, but no study was made of these.

Higher temperatures and higher light intensities gave quicker and more intense staining of root tips. Bright light (direct sunlight) and higher temperatures (37-40° C.) affect the reduction strongly so that the material stains almost immediately. Daylight from blue sky was superior to laboratory light. Root tips heated at 60° C. did not stain at all. When the pH of a tetrazolium solution was increased from 5 to 8 or 9 with potassium hydroxide the reaction was very quick and comparable to the reaction in direct sunlight.

Brown (11) stated that it is doubtful that tetrazolium reduction should be considered a specific test for reducing enzymes. Matteon *et al.* (61) concluded that in all probability the reduction of tetrazolium compounds by enzymes of living cells cannot be

considered a general test for life. Nevertheless, the unusual properties of these reagents suggest that they might be utilized in many types of biological research involving differences in tissue viability.

III. TOLERANCES OF CITRUS SEEDLINGS TO FREE WATER IN LEON FINE SAND

No research data are available on the behavior of citrus seedlings in flooded soil of any specific type. Because of this and the present interest in the planting of citrus on soils which are classified as poorly drained, experiments were conducted to study the response of citrus seedlings, used as rootstocks in Florida, to standing free water in soil. Citrus seedlings were flooded during different months of the year in the greenhouse. Leon fine sand was the soil type used because it is the predominant flatwoods soil type available for citrus.

Methods and Results

The soil used in this study came from a native pasture covered with palmetto, gallberry and wire grass in Polk County, Florida, and was Leon fine sand, which is a somewhat poorly drained soil with a hardpan. The description of the soil has been given elsewhere (55). The profile was arbitrarily divided into two horizons, topsoil 0-8" and subsoil 8-17" (leached layer on top of the hardpan), both of which were used for growing the transplanted seedlings in metal cans prior to flooding.

The kinds of citrus used in the flooding experiments were: Rough lemon (RL), Citrus limon; (variety unknown) sour orange (SO), C. aurantium; Pineapple sweet orange (SwO), C. sinensis; Cleopatra mandarin (Cleo), C. reticulata; Troyer citrange (Troyer) Poncirus trifoliata x C. sinensis; Rusk citrange (Rusk) P. trifoliata x C. sinensis; and Carrizo citrange (Carrizo), P. trifoliata x C. sinensis. The seeds were germinated in a sand-peat medium in flats in the greenhouse in 1957 and 1956 at the University of Florida Citrus Experiment Station. The seedlings were fertilized with a complete nutrient solution every month and watered once a week.

Experiment 1.- Flooding citrus seedlings in a tank of water.

Twenty one-year-old sweet orange seedlings and 8 one-year-old Rough lemon seedlings were transplanted into 46-ounce metal cans which were filled with Leon topsoil in July, 1957. Holes were punched in the cans at the bottom to facilitate drainage. Two months after these seedlings were transplanted they were placed in a galvanized tank in the greenhouse and the tank was filled with water so that the water level was above the top of each can. During the flooding period the temperature of the water fluctuated between 75° F. and 95° F.

After 3 weeks in the flood tank only 1 sweet orange seedling was wilting. All of the seedlinge were then removed and the cans with the sweet orange eeedlinge were divided into 3 groupe. One group of 6 cans wae placed in the greenhouee, the eecond group of 6 cans wae placed in shade outside the greenhouse, and the third group of 5 cane was placed in the open sunlight outside the greenhouee. The other 3 eeedlinge had been removed for observation. Six oane with Rough lemon seedlinge were left in the greenhouse after they were removed from the flood tank. No water was added to the cans for 3 weeks at which time only the seedlings in the open sunlight had wilted. The soil in theee cane was dry. There were new growth and new roote on all of the non-wilted eeedlings.

Six, one-year-old sweet orange eeedlinge in Leon topsoil in 46-ounoe metal cans were individually placed in eteel oontainere in a tank of water which wae thermo-etatically controlled at 72° F. The cans and oontainers were filled with water and the surface of the soil was covered with aluminum foil. The cans containing the seedlings had drain holee in the bottom, neoessitating a second container which was surrounded by the water in the tank. For 6 weeks during October and November the seedlinge remained green and turgid.

Experiment 2.- Citrus seedlings flooded in January, 1958.

Forty 46-ounce metal cans were filled with topsoil (pH 4.5). These cans were divided into 4 groups of 10 each and 4 seedlings of 1 variety were transplanted into each of the 10 cans. The 4 varieties transplanted were Rough lemon, sour orange, sweet orange and Cleopatra mandarin; they remained in the greenhouse. Nine of the 10 cans of each variety were flooded in January, 2 months after the seedlings had been transplanted. The remaining cans were watered once a week as a control. There were no drain holes in the cans, therefore the water in the cans became stagnant, but fresh water was added when needed to maintain the water level above the soil surface. Four flooded cans, 1 each with Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings, were emptied and the root systems of all the seedlings were examined after 2, 4, 6, 9, 14, 21 and 39 days of flooding. The other flooded cans, 2 of each variety, remained flooded for 1 year.

None of the seedlings, regardless of the length of time in the flooded soil, showed any wilting symptoms. There were no sloughed roots on any of the varieties after 14 days of flooding. Less than one-tenth of the root tips of each seedling were sloughed after 21 days

of flooding. The lower half of the root syetem and approximately 100 per cent of the root tips on all of the seedlings were rotted after 39 days of flooding. There were 1 or 2 new roots on each of the Rough lemon, sour orange and Cleopatra mandarin seedlings which had been flooded for 39 days. These new roots were from the tap root near the base of the stem just below the soil surface. The yellowing of the leaves was slightly more pronounced on the flooded seedlings after 1 year than on the non-flooded seedlings. There were well-distributed new root systems on all of the seedlings that were flooded for 1 year. None of the original feeder roots was present, but about 2 inches of the tap root did remain and it was from this portion that the new roots originated.

Experiment 3.- Citrus seedlings flooded in April, June, July and August, 1958.

Thirty 46-ounce metal cans were filled with topsoil and 30 cans were filled with subsoil. Four seedlings, 1 each of Rough lemon, sour orange, Cleopatra mandarin and sweet orange, were transplanted into each can, and watered with 100 ml of water once or twice weekly as needed. Ten of the cans with the topsoil and seedlings and 10 cans with the subsoil and seedlings

were flooded on April 4, five months after the seedlings were transplanted.

Also, on each of the following dates, June 1, July 1, and August 7, six of the cans with topsoil and 6 with subsoil were flooded. The water remained standing in each can following its flooding for the duration of the flooding periods. Additional water was added when needed to keep the water level above the soil surface.

The first visible symptom of injury on any of the seedlings that were flooded in April occurred 5 weeks later. A numerical rating was given to the various symptoms observed during this and the other periods of prolonged flooding:

0 - Leaves green and turgid.

1 - Leaves yellow-veined and turgid.

2 - Leaves wilted (either drooped or rolled).

3 - Leaves defoliated or desiccated.

In order to compare the tolerance of the different citrus seedlings to standing free water, the weekly index rating for each variety in each soil was calculated by multiplying the number of seedlings in each category by their respective numerical rating (numbers from 0 to 3), adding the products, and then dividing the summed products by the total number of seedlings. The results

for the seedlings flooded in April are presented in Figures 1 and 2 and Table 1.

Analysis of variance showed that there was a highly significant difference between varieties, weeks and soils (subsoil and topsoil). There was no significance between the interactions of any of these components. The seedlings in the flooded subsoil were injured to a significantly higher degree than those in the flooded topsoil (Figure 1). The Rough lemon seedlings were more tolerant of the water than the sour orange, sweet orange or Cleopatra mandarin seedlings (Figure 2). The sweet orange and Cleopatra mandarin seedlings were more tolerant of the free water than the sour orange. There was no significant difference between the sweet orange and Cleopatra mandarin seedlings in their tolerance of the free water. Therefore, the order of tolerance of the citrus seedlings to free water in Leon soil when flooded in April in cane was Rough lemon, sweet orange, Cleopatra mandarin and sour orange. As was expected, there was a highly significant difference between the weeks (5-10) although there was no difference in the appearance of the seedlings until 5 weeks after flooding. The citrus seedlings which were flooded in June, July and August showed water injury symptoms within 2 weeks after the cane were flooded. The weekly index ratings

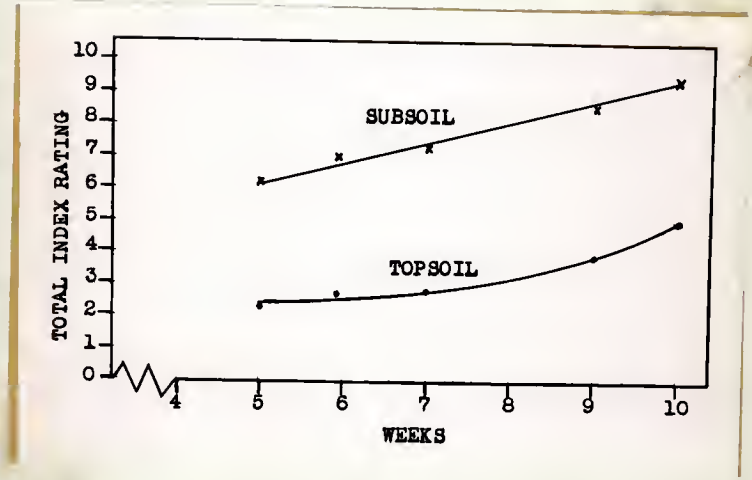

Figure 1.- Total index rating of injury to citrus seedlings in Leon topsoil and subsoil during ten weeks of continual flooding from April 1.

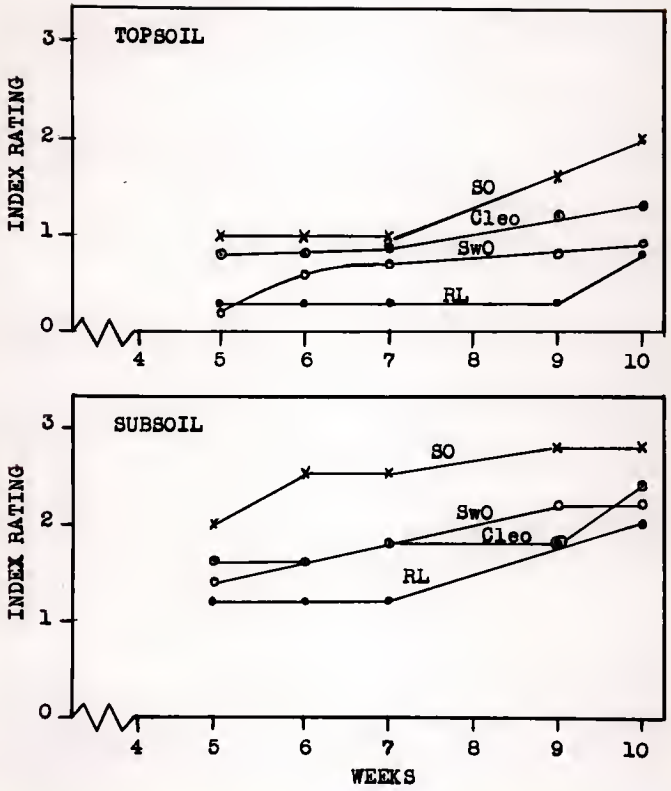

Figure 2.- Index rating of injury to Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings in Leon soil during ten weeks of continual flooding from April 1.

TABLE 1.- Index rating of injury to Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings when flooded in Leon subsoil during April, 1958.

Variety	Weeks 5		6		7		9		10		Totals		Total
	Soil* T	S	T	S	T	S	T	S	T	S	T	S	
RL	0.3	1.2	0.3	1.2	0.3	1.1	0.3	1.8	0.8	2.0	2.0	7.3	9.3
SO	1.0	2.0	1.0	2.6	0.9	2.6	1.6	2.8	2.0	2.8	6.5	12.8	19.3
SwO	0.2	1.4	0.6	1.6	0.7	1.8	0.8	2.2	0.9	2.2	3.2	9.2	12.4
Cleo	0.8	1.6	0.8	1.6	0.9	1.8	1.2	1.8	1.3	2.4	5.0	9.2	14.2
Total	2.3	6.2	2.7	7.0	2.8	7.3	3.9	8.6	5.0	9.4	16.7	38.5	55.2

* T- topsoil
S- subsoil

Varieties: LSD at 0.05 is 2.14
LSD at 0.01 is 2.99

Soil: LSD at 0.01 is 3.06

TABLE 1-Continued

Analysis of Variance

Source of Variation	d.f.	S.S.	M.S.	F.	Required F .05	Required F .01
Total	39	21.08				
Varieties (V)	3	5.26	1.75	36.45	3.49	5.95
Weeks (W)	4	2.82	0.71	14.68	3.26	5.41
Soil (S)	1	11.88	11.88	247.50	4.75	9.33
V x W	12	0.24	0.02	0.42	2.69	4.16
V x S	3	0.26	0.09	1.81	3.49	5.95
W x S	4	0.04	0.01	0.21	3.26	5.41
V x W x S	12	0.58	0.05			

for the flooded citrus seedlings are presented in Figures 3 and 4 and in Table 2.

Analysis of variance showed that there was a highly significant difference between varieties in the over-all flooding of the soils. By the t-test the Rough lemon seedlings were significantly more tolerant of the free water than the sweet orange seedlings; the Cleopatra mandarin seedlings were significantly more tolerant of free water than the sour orange and sweet orange seedlings. There was no difference between varieties in the subsoil. However, both the Rough lemon and Cleopatra mandarin seedlings were significantly more tolerant of the flooded topsoil than the sour orange or sweet orange seedlings, with no significant difference between the Rough lemon and Cleopatra mandarin seedlings.

There was a highly significant difference in the water tolerance of the citrus seedlings in the topsoil and in the subsoil, between the weeks (1-4) and between the months (June, July and August). This is illustrated in Figure 3. Other highly significant differences were between the interactions of varieties and soils, weeks and soils, and weeks and months. After 2 weeks of flooding the seedlings in the subsoil were much more severely injured than those in the topsoil, which is shown by the highly significant difference of the

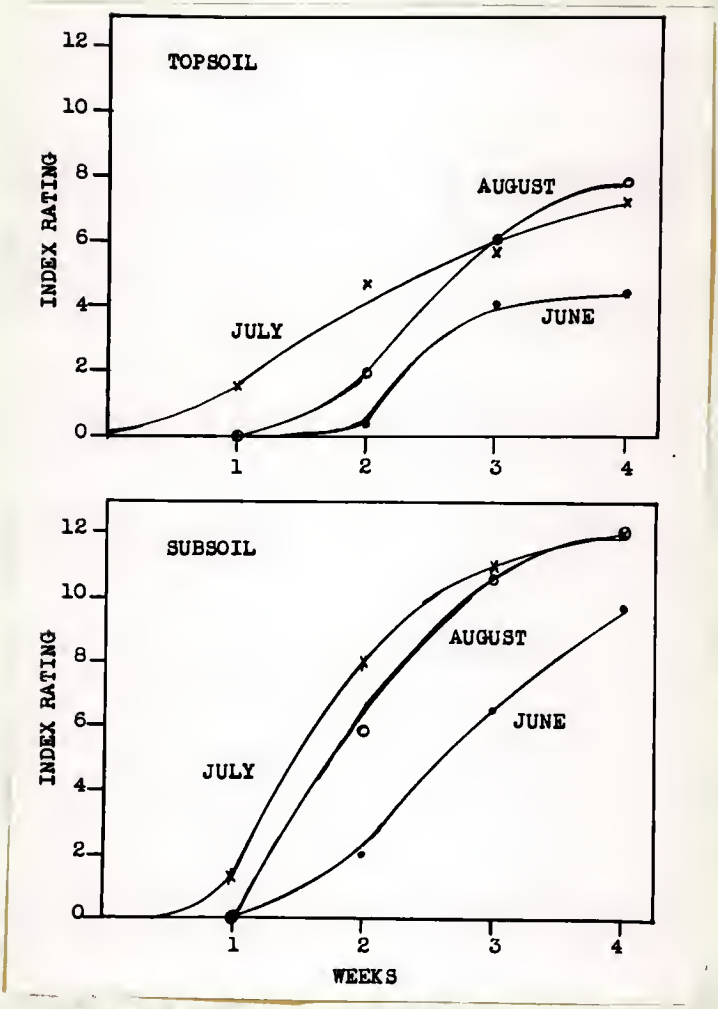

Figure 3.- Total index rating of injury to citrus seedlings in Leon soil during four weeks of continual flooding in the months of June, July and August.

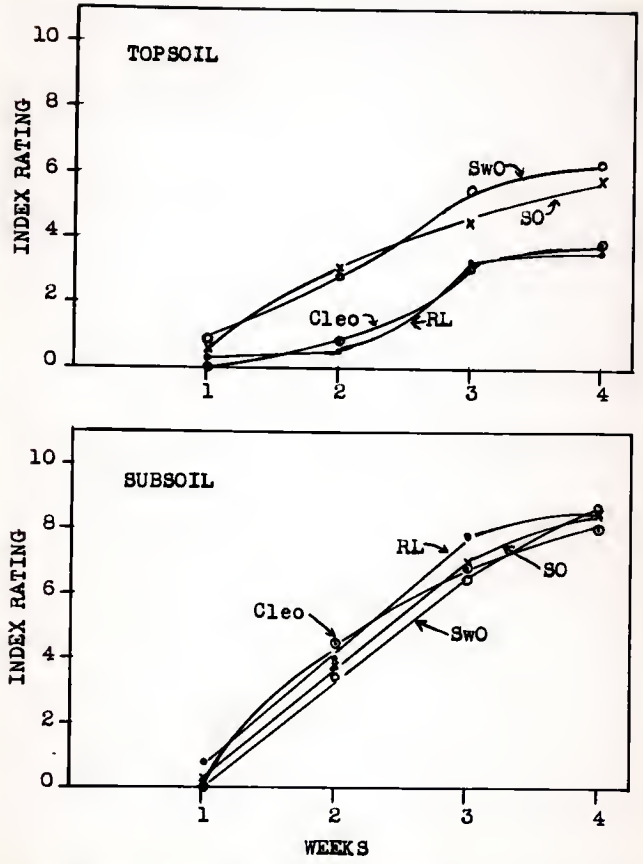

Figure 4.- Index rating of injury to Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings in Leon soil during four weeks of continual flooding in June, July and August.

TABLE 2.- Index rating of injury to citrus seedlings when flooded in Leon topsoil and subsoil during June, July and August, 1958.

Variety	Weeks																								
	1						2				3								4						
	Months																								
	June		July		Aug.		June		July		Aug.		June		July		Aug.		June		July		Aug.		
	Soil*																								
	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	T	S	Grand Total
RL	0	0	0.33	0.66	0	0	0	0.33	0.50	1.83	0	1.83	1.00	1.83	0.83	3.00	1.33	3.00	1.00	2.50	1.33	3.00	1.33	3.00	7.65	20.98	28.63
BO	0	0	0.50	0.33	0	0	0.33	0.66	1.66	2.00	1.16	1.16	0.83	1.16	1.83	3.00	1.83	2.83	1.16	2.50	2.33	3.00	2.33	3.00	13.96	19.64	33.60
BwO	0	0	0.83	0.16	0	0	0.16	0.50	1.83	2.00	0.83	1.00	1.83	1.50	2.00	2.66	1.66	2.33	1.83	2.66	2.16	3.00	2.33	3.00	15.46	18.81	34.27
Cleo	0	0	0	0.16	0	0	0	0.50	0.50	2.13	0.33	1.83	0.50	2.00	1.00	2.33	1.50	2.50	0.50	2.00	1.50	3.00	1.83	3.00	7.66	19.45	27.11
Total	0	0	1.66	1.31	0	0	0.49	1.99	4.49	7.96	2.32	5.82	4.16	6.49	5.66	10.99	6.32	10.66	4.49	9.66	7.32	12.00	7.82	12.00	44.73	78.88	123.61

* T- topsoil
S- subsoil

Variety totals: LSD at 0.05 is 3.33
LSD at 0.01 is 4.56

Varieties within soils: LSD at 0.05 is 2.35
LSD at 0.01 is 3.22

TABLE 2-Continued

Analysis of Variance

Source of Variation	d.f.	S.S.	M.S.	F.	Required F .05	Required F .01
Total	95	103.53				
Variety (V)	3	1.59	0.53	10.16	3.16	5.09
Weeks (W)	3	63.40	21.14	404.86	3.16	5.09
Soil (S)	1	12.15	12.15	232.72	4.41	8.28
Months (M)	2	9.74	4.87	93.27	3.55	6.01
V x W	9	0.73	0.08	1.56	2.46	3.60
V x S	3	2.86	0.95	18.25	3.16	5.09
V x M	6	0.76	0.13	2.44	2.66	4.01
W x S	3	5.05	1.68	32.23	3.16	5.09
W x M	6	2.89	0.48	9.21	2.66	4.01
S x M	2	0.28	0.14	2.74	3.55	6.01
V x W x S	9	0.62	0.07	1.33	2.46	3.60
V x W x M	18	1.27	0.07	1.35	2.19	3.07
W x S x M	6	0.70	0.12	2.23	2.66	4.01
V x S x M	6	0.55	0.09	1.75	2.66	4.01
V x W x M x S	18	0.94	0.05			

interaction between the soils and weeks. This is graphically shown in Figure 4. After 2 weeks of flooding the seedlings floodsd in July had a higher indsx rating than thoee flooded for the eame period of time in Juns or Auguet. At ths end of 3 and 4 weeke of flooding the index rating for the seedlinge flooded in July and August were approximately the same and higher than the index rating for ths ssedlinge flooded in June for the sams length of time. Thie offers an sxplanation for ths highly significant difference of ths intsraction between weeks and months.

The avsrage monthly maximum and minimum tempsratures recorded at the Unitsd Statss Weather Climatological Station 4707, Lake Alfred, Florida, for the months in which ths seedlings were flooded ars as follows: (maximum, minimum respectively) January 65,44; February 65,41; March 74,54; April 82,60; May 85,65; June 91,71; July 92,73; August 92,73; September 92,72.

Experiment 4.- Effect of lime on citrue eeedlinge in flooded eoil.

Ssven-month-old esedlinge of Rough lemon, eour orange, sweet orange, Cleopatra mandarin and Troyer citrangs wers transplanted into 46-ounce metal cans which were filled with Leon subsoil. The subeoil was used becaues of ths reeults from ths April flooding. There was 1 seedling

per can and 20 cans of each variety in the virgin soil. Also, 20 seedlings of each variety were transplanted into cans filled with subsoil to which dolomitic limestone had been added at the rate of 6000 pounds per acre at the time of transplanting. There were 12 Carrizo citrange seedlings in each of the limed and unlimed soils, 10 Rusk citrange seedlings in the limed soil, and 7 in the unlimed soil. The Carrizo and Rusk citrange seedlings were 18 months old at the time they were transplanted. The transplanted seedlings were grown in the greenhouse for 6 weeks before they were flooded. During this period the cans were watered once a week with approximately 150 ml of deionized water. The pH of the soil was determined at the time the seedlings were transplanted, before they were flooded, and after 4 weeks of continual flooding.

The seedlings were flooded on July 22, 1958, in the greenhouse. Water was added to the cans when needed to keep a water level above the soil surface. A weekly numerical rating of various symptoms was made for 6 weeks of continual flooding for each seedling. The weekly index ratings were made in a similar manner as in Experiment 2 using the same rating system for all seedlings of each variety in both limed and unlimed soil. The results are presented in Table 3. An analysis of

TABLE 3.- Index rating of injury to Rough lemon, sour orange, sweet orange, Cleopatra mandarin, Troyer, Carrizo and Rusk seedlings when flooded in Leon subsoil with and without dolomitic limestone.

Variety	Weeks														
	1		2		3		4		5	6					
	Lime Treatment														
	lime	no lime	lime	no lime	lime	no lime	lime	no lime	lime	no lime	lime	no lime	Totals lime	Totals no lime	Grand Total
RL	0	0.45	0	1.3	0	1.6	0.05	2.10	0.10	2.3	0.55	2.45	0.70	70.20	10.90
SO	0	0.60	0	1.2	0	1.0	0	1.20	0.15	1.4	0.85	1.25	1.00	6.65	7.65
SwO	0	0.80	0	1.75	0	1.85	1.40	2.45	1.30	2.7	1.60	2.75	4.30	12.30	16.60
Cleo	0	1.20	0	2.00	0	2.15	0.55	2.55	1.05	2.75	1.20	2.80	2.80	13.45	16.65
Troyer	0	0	0	0.15	0	0.75	0	0.90	0	0.85	0	0.95	0	3.60	3.60
Carrizo	0	0	0	0.50	0	1.00	0	1.16	0	1.41	0	1.41	0	5.48	5.48
Rusk	0	0	0	2.00	0.42	2.80	1.43	2.80	1.70	2.90	1.86	2.90	5.41	13.40	18.81
Total	0	3.05	0	8.90	0.42	11.15	3.43	13.16	4.30	14.31	6.06	14.51	14.21	65.08	79.29

Variety totals: LSD at 0.05 is 2.74
LSD at 0.01 is 3.69

Varieties within treatment: LSD at 0.05 is 1.93
LSD at 0.01 is 2.60

TABLE 3-Continued

Analysis of Variance

Source of Variation	d.f.	S.S.	M.S.	F	Required F .05	.01
Total	83	77.80				
Time (T)	5	15.66	3.13	41.87	2.53	3.70
Lime (L)	1	30.81	30.81	411.85	4.17	7.56
Varieties (V)	6	17.97	3.00	40.04	2.42	3.47
V x L	6	3.06	0.51	6.82	2.42	3.47
T x L	5	2.76	0.55	7.38	2.53	3.70
V x T	30	5.30	0.18	2.36	1.84	2.38
V x T x L	30	2.24	0.07			

variance was made to determine any significant differences.

The pH of the soil at the time the seedlings were transplanted was 4.6. Six weeks after the dolomitic limestone had been added to the soil and just prior to flooding the pH of the limed soil had increased to 7.5. The pH of the limed soil with citrus seedlings was 7.0 after 4 weeks of flooding, but was not changed without seedlings. The unlimed flooded soil with citrus seedlings had a pH of 5.0 after 4 weeks of flooding and a pH of 4.0 without citrus seedlings.

There was a highly significant difference between the degree of injury to the seedlings of each variety in the limed soil and the unlimed soil for the 6 weeks of flooding. The injury to the seedlings in the unlimed soil was progressively more than the injury to the seedlings in the limed soil. There was no difference between the rating of the seedlings of the 7 varieties in the limed soil after 3 weeks of flooding. In the unlimed flooded soil during the first 3 weeks of flooding the least injured varieties to the most injured were: Troyer citrange, Carrizo citrange, sour orange, Rough lemon, sweet orange, Cleopatra mandarin and Rusk citrange. After 4 weeks of continual flooding the Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings were photographed to illustrate the difference between ths

condition of the tops of the plants in the unlimed and limed soil (see Figures 5, 7, 9, 11). Figures 6, 8, 10, 12 and 13 illustrate the change in the condition of the seedlings (in the limed and unlimed soil) during the 6 weeks of continual flooding. It is possible for one to look at the graph accompanying each picture and compare the index rating of all the seedlings after 4 weeks of continual flooding with the representative seedlings in the photograph.

After 6 weeks of continual flooding there were significant differences in the injury index rating between the Rough lemon, sweet orange, Cleopatra mandarin, and Rusk citrange seedlings; the sour orange, sweet orange, and Rusk citrange seedlings; and the Cleopatra mandarin and Rusk citrange seedlings in the limed soil. In the unlimed soil there was no significant difference between the sweet orange, Cleopatra mandarin and Rusk citrange seedlings, and the sour orange and Carrizo citrange seedlings, whereas there were significant differences among all of the other seedlings.

Experiment 5.- Effect of carbon dioxide.

One-gallon metal cans were filled to within 2 inches of the top with Leon subsoil. A glass tube with a fine mesh wire over the bottom was placed in the center of each can at the time the cans were filled with the soil

Figure 5.- Rough lemon seedlings in Leon subsoil with (right) and without (left) dolomite following four weeks of continual flooding.

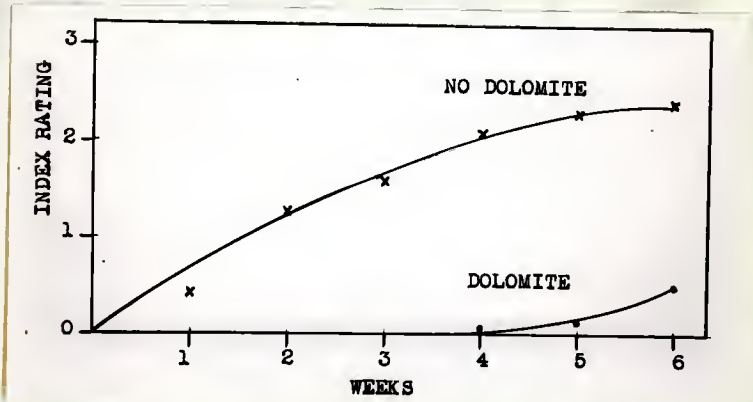

Figure 6.- Index rating of Rough lemon seedlings in Leon subsoil with and without dolomite during six weeks of continual flooding.

Figure 7.- Sour orange seedlings in Leon subsoil with (right) and without (left) dolomite following four weeks of continual flooding.

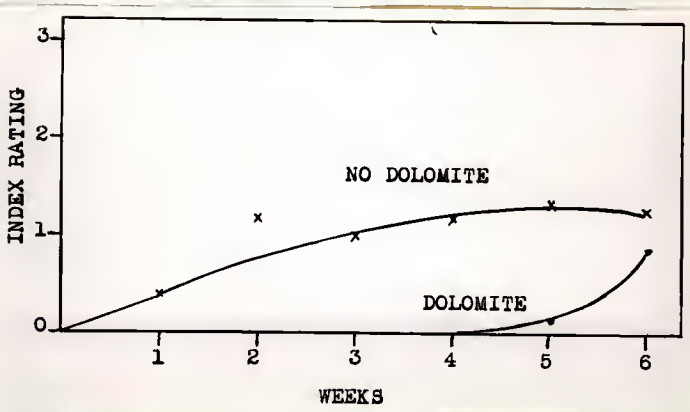

Figure 8.- Index rating of injury to sour orange seedlings in Leon subsoil with and without dolomite during six weeks of continual flooding.

Figure 9.- Sweet orange seedlings in Leon subsoil with (right) and without (left) dolomite following four weeks of continual flooding.

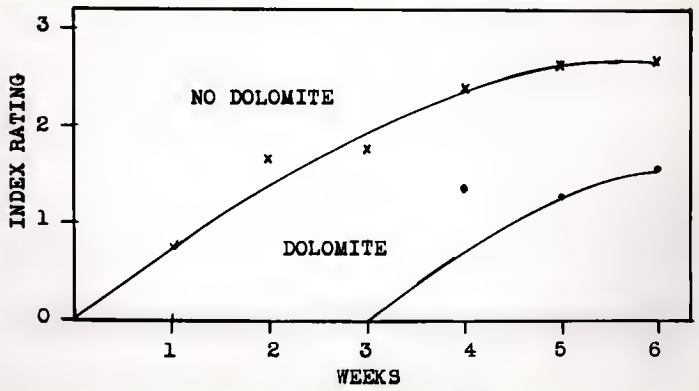

Figure 10.- Index rating of injury to sweet orange seedlings in Leon subsoil with and without dolomite during six weeks of continual flooding.

Figure 11.- Cleopatra mandarin seedlings in Leon subsoil with (right) and without (left) dolomite following four weeks of continual flooding.

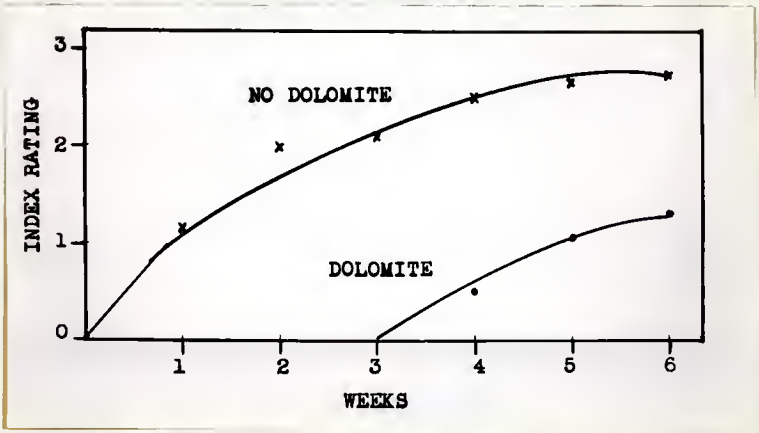

Figure 12.- Index rating of injury to Cleopatra mandarin seedlings in Leon subsoil with and without dolomite during six weeks of continual flooding.

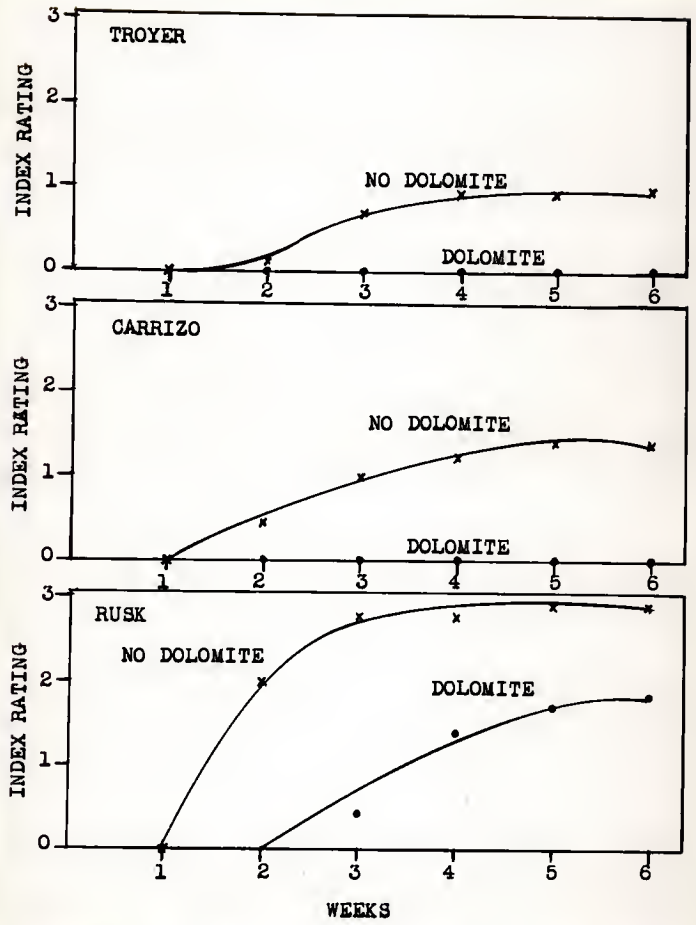

Figure 13.- Index rating of injury to Rusk, Troyer and Carrizo citranges in Leon subsoil with and without dolomite during six weeks of continual flooding.

and 6 seedlings of each variety were planted per can. The varieties of seedlings used were Rough lemon, sour orange, Cleopatra mandarin and sweet orange. There were 3 cans of each variety. Two cans of each variety were flooded with deionized water in June, 1958, three months after they were transplanted. Compressed carbon dioxide was slowly released into one flooded can and one unflooded can of each variety through the glass tube for the first 24 hours the cans were flooded. During the succeeding 8 days carbon dioxide was released into the cans for 15 hours each day. The injury symptoms of each plant were rated using the rating system of Experiment 2 and an index rating for all the seedlings of each variety with and without carbon dioxide was made the same as that in the above experiment.

The index rating of the seedlings in the flooded soil with and without added carbon dioxide is presented in Table 4. After 1 week of flooding there was no difference between any of the seedlings in the flooded or non-flooded cans with or without additional carbon dioxide. After 2 weeks of flooding the flooded seedlings with additional carbon dioxide had a higher index rating of injury than the flooded seedlings without additional carbon dioxide. This relation continued for 5 weeks. There was a highly significant difference between the

TABLE 4.- Index rating of injury to Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings in Leon subsoil with and without added carbon dioxide.

Variety	Weeks												
	1		2		3		4		5		Total		Grand Total
	Treatment												
	CO_2	No CO_2	CO_2	No CO_2	CO_2	No CO_2	CO_2	No CO_2	CO_2	No CO_2	CO_2	No CO_2	
RL	0	0	1.00	0	1.83	0	3.00	1.00	3.00	2.33	8.83	3.33	12.16
SO	0	0	0.66	0	1.66	0	3.00	1.00	3.00	1.50	8.32	2.50	10.82
SwO	0	0	0.66	0	3.00	0.60	3.00	1.20	3.00	2.00	9.66	3.80	13.46
Cleo	0	0	1.00	1.00	2.00	2.50	3.00	2.50	3.00	2.50	9.00	8.50	17.50
Total	0	0	3.32	1.00	8.49	3.10	12.00	5.70	12.00	8.33	35.81	18.13	53.94

Varieties within treatment: LSD at 0.05 is 2.59
LSD at 0.01 is 3.64

Variety totals: LSD at 0.05 is 3.67
LSD at 0.01 is 5.15

Between treatment totals: LSD at 0.01 is 7.27

TABLE 4-Continued

Analysis of Variance

Source of Variation	d.f.	S.S.	M.S.	F	Required F .05	.01
Total (T)	39	56.46				
Varieties (V)	3	2.50	0.83	5.88	3.49	5.95
Weeks (W)	4	37.21	9.30	65.65	3.26	5.41
CO_2 (C)	1	7.81	7.81	55.12	4.75	9.33
V x W	12	2.04	0.17	1.20	2.69	4.16
V x C	3	2.06	0.69	4.85	3.49	5.95
W x C	4	3.14	0.79	5.54	3.26	5.41
V x W x C	12	1.70	0.14			

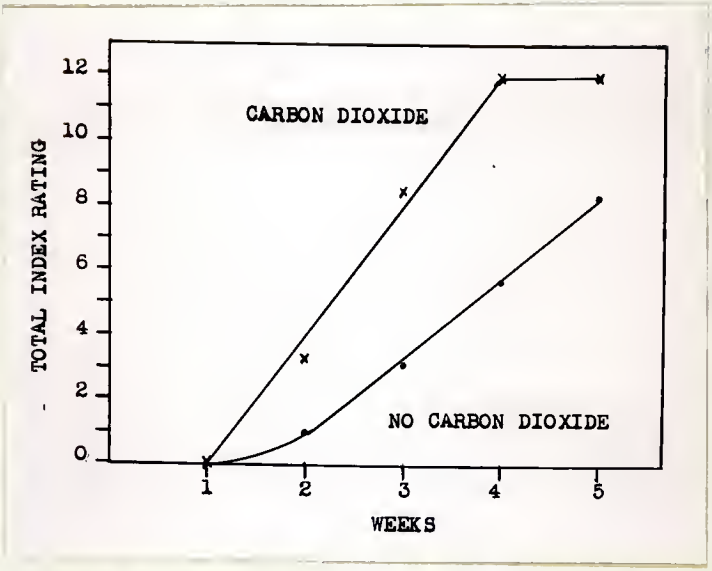

Figure 14.- Total index rating of injury to citrus seedlings in Leon subsoil with and without added carbon dioxide.

flooded seedlings with and without carbon dioxide added (see Figure 14). The only significant difference between varieties was between the Cleopatra mandarin seedlings and the Rough lemon, sour orange, and sweet orange seedlings in the flooded soil without additional carbon dioxide. This is shown in Figure 15. There was no significant difference between any of the varieties in the flooded soil in which carbon dioxide was added.

The seedlings in the unflooded cans to which carbon dioxide was added never wilted. Two weeks after the carbon dioxide was first introduced there was new growth present on 80 per cent of the seedlings.

Discussion

When the root system of a plant has been injured in water-saturated soils the top will eventually show injury. The first visible sign on citrus seedlings in these experiments was a yellowing of the leaf veins. The leaf pattern as shown in Figure 16 was more outstanding on sour orange and sweet orange. The symptom first appeared on the lower leaves of the seedlings. Bain and Chapman (2) observed this same condition on grapefruit plants in water-saturated soils. The next symptom of injury was wilting of the leaves. The young tender leaves wilted first. The leaves when wilted were either drooped or curled. After the seedlings wilted the leaves sometimes

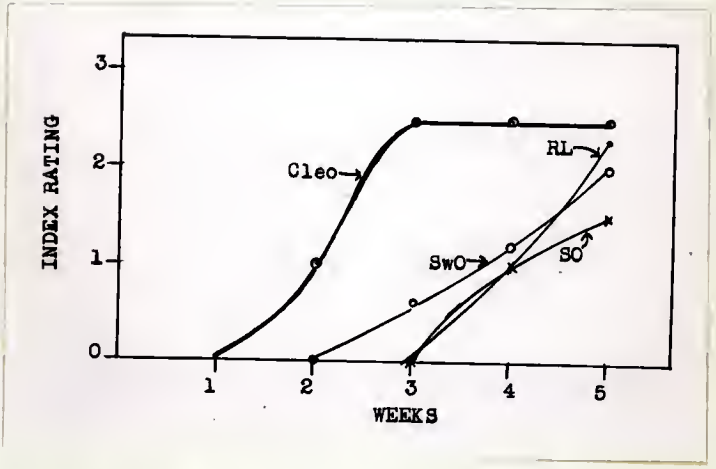

Figure 15.- Comparative index ratings of injury to Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings in flooded Leon subsoil without added carbon dioxide.

Figure 16.- Typical yellow-veined pattern (left) in leaves of a sour orange seedling indicating early injury symptoms following extended flooding compared with a healthy green seedling (right).

defoliated before desiccating or they appeared desiccated and remained tightly on the stems. These symptoms were a result of injury to the root system which may vary in a seedling from the loss of many of the fibrous feeding roots to the death of the tap root. In larger plants having a more branched root system, death of both small and large woody roots, in addition to the loss of the fibrous roots, and rotting of the root crowns may be manifested in the top of the tree by reduced growth. Smallness, sparseness, and yellowing of the foliage and more or less complete defoliation will ensue.

Sweet orange and Rough lemon seedlings in water-saturated soil in a water tank survived flooding for 3 weeks during the month of September. More than 75 per cent of the sweet orange seedlings that were flooded in cans in July and August but not in a water bath were wilted after 2 weeks. The temperature range (75° F. to 95° F. each day) was comparable. There was a prolific growth of algae in the water in the tank in which the seedlings were flooded which undoubtedly increased the oxygen content of the water. Since the cans had holes in them there was an interchange of water in the cans with the water in the tank, whereas there was no such interchange of water in the flooded cans outside of

the water tank. The water in the cans not in the water tank was stagnant and had an odor of putrefaction. In the flooded cans accumulation of toxic substances as proposed by Kramer (50) or Jackson (38) would be far greater than in the cans in the water tank. If toxic substances had been formed in the water tank they could have become diluted to such an extent that they were not harmful to the plant or they could have become oxidized as a result of increased oxygen due to algal growth. The sweet orange seedlings, in a water-saturated soil at a constant temperature with the soil-water surface covered to eliminate light, were tolerant of the water for six weeks. In this case the dilution factor was the most probable reason for the elimination of high concentrations of any toxin which might have formed.

Four varieties of citrus seedlings tolerated water-saturated soil for one year when flooded in January. No extensive root injury was observed during the first 39 days of flooding. There was a complete new root system on all seedlings in cans which were flooded for one year and the old root system had completely decayed. Apparently the new root system was formed during the time which the plants were under minimum transpirational stress. When the plants became more active they

were dependent upon the new root system which was adapted to the flooded environment.

When seedlings were flooded in the summer months, plants were in a more active stage of growth and their leaves displayed injury symptoms within 2 weeks. During the spring and summer of 1958, the seedlings flooded in July were damaged sooner as evidenced by the leaf symptoms. Injury symptoms appeared sooner during June, July and August than in April. This could be due to variances in temperature resulting in a differential rate of transpiration. Heinicke (34) found that flooding the soil containing apple roots during the winter months caused considerable loss of small roots but produced no serious injury if the soil was drained before leaves began to appear. Flooding in the summer soon caused injury, particularly if transpiration was rapid.

There was a striking difference between the degree of injury to the citrus seedlings in flooded subsoil and flooded topsoil. Soil from both depths had a comparable pH of 4.5. The higher organic matter content in the topsoil could influence the water tolerance of these seedlings, either by a physical or chemical reaction with substances that caused injury, be they organic or inorganic. The subsoil was almost devoid of organic matter.

When a soil is flooded in the field and the water is removed only by natural factors the subsoil retains the water longer than the topsoil. Citrus trees may suffer from decided rises of the water table during the rainy season of the year, especially when the occurrence of one or more fairly dry years has induced the root systems to extend themselves fairly deeply into the subsoil. It is in this horizon where the root injury is most likely to occur first and most severely. If water becomes stagnant more injury is likely to be shown by the tops of the plants than if water is moving. Moving water will remove or dilute toxic accumulations from the root areas. Water from the surface of the soil can be removed by evaporation or run off, and sooner than water in the subsoil, thus allowing subsoil water to become more stagnant and more harmful to the roots and to the entire plant. Reitz and Long (71) found that in poorly drained areas approximately 75 per cent of the root system of the citrus trees was located in the surface 12-inch level.

Plants in the limed soil tolerated the waterlogged condition for a longer time than the plants in the acid soil. There was sufficient time for the reaction of the lime with the soil before flooding to raise the pH of the soil to near neutral. Besides the pH change additional cations were present. The reason for the

influence this change had on the water tolerance of the citrus plants is uncertain. If roots of citrus secrete a substance with or without the aid of microorganisms when flooded and that substance can be changed chemically by the presence of cations at the higher pH, the seedlings might tolerate excess water for a longer period of time instead of collapsing within a short time as was the case in the acid medium. Truog (88) proposed that an equilibrium condition, chemically and physically, exists between toxic substances in the soil solution and the solid soil constituents. They may combine directly or react by double decomposition with these constituents. Thus the equilibrium concentration is disturbed.

New roots were present on Rough lemon, sweet orange and Cleopatra mandarin seedlings in the limed soil but not on the seedlings in the unlimed soil. These differences are shown in Figures 17-19 inclusive. The new roots are white and near the base of the stem. New roots were formed on sour orange and Troyer citrange seedlings in both the limed and unlimed soil (Figures 20 and 21). The rapid collapse of the root systems in the acid soil was reflected in a relatively short time in the tops and this damage to the tops probably accounts for the absence of new roots. Even though the old root

Figure 17.- Typical Rough lemon root systems following six weeks of continual flooding in Leon subsoil with (left) and without (right) dolomite.

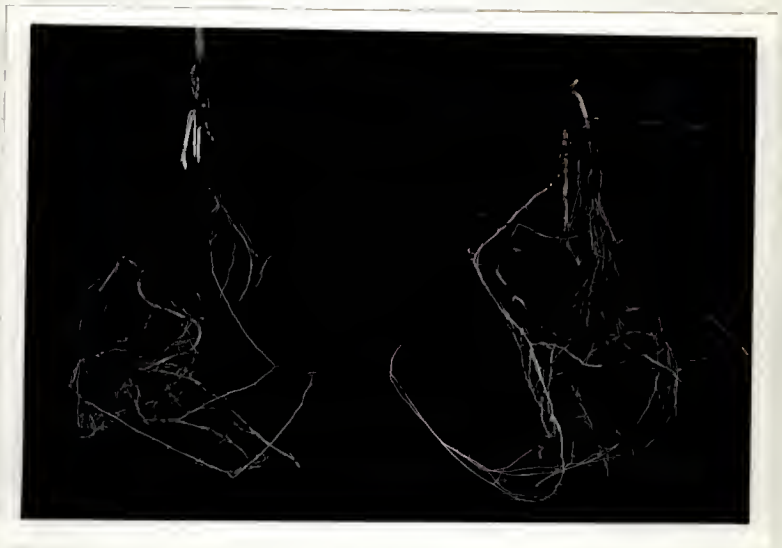

Figure 18.- Typical sweet orange root systems following six weeks of continual flooding in Leon subsoil with (left) and without (right) dolomite.

Figure 19.- Typical Cleopatra mandarin root systems following six weeks of continual flooding in Leon subsoil with (left) and without (right) dolomite.

Figure 20.- Typical sour orange root systems following six weeks of continual flooding in Leon subsoil with (left) and without (right) dolomite.

Figure 21.- Typical Troyer citrange root systems following six weeks of continual flooding in Leon subsoil with (left) and without (right) dolomite.

systems on the plants in the neutral soil had been injured it could have been a more gradual injury and new roots began to form which aided in the support of the tops. Rough lemon seedlings in the limed soil had the largest new root system with the Troyer citrange seedlings next. The root systems on the Carrizo citrange seedlings were comparable to those on the Troyer citrange seedlings. Both regenerated new roots in the acid and limed flooded soil. In all of the experiments where seedlings were flooded for extended periods of time any seedlings which survived after 6 weeks of flooding had new roots near the base of the stem just below the soil surface. These newly formed roots undoubtedly become a factor in the survival of the plants under flooded conditions. A soil medium conducive to better root function seems to be conducive to greater water tolerances.

Carbon dioxide, when added to the flooded soil with citrus seedlings, caused earlier water injury symptoms to appear in the leavee than where no carbon dioxide was added. Also these seedlinge that were treated with carbon dioxide desiccated earlier. The carbon dioxide apparently caused earlier root injury which was reflected by the leavee. It is believed (25) that the specific effect could be on the protoplasm, causing an increase in viscosity and decrease in

permeability, and this would affect the water absorption mechanism rather than affect transpiration. Cannon (12) found that citrus roots could tolerate high concentrations of carbon dioxide in a porous medium not waterlogged. No apparent damage was done to the seedlings in the unflooded soil to which carbon dioxide was added; indeed, new growth appeared 1 week after the carbon dioxide treatment ceased. It seems to the author that carbon dioxide probably injures the roots but with water present other factors are involved which are stimulated by additional carbon dioxide.

In flooded acid soil with one seedling in each can, the order of water tolerance was: Troyer citrange, Carrizo citrange, sour orange, Rough lemon, sweet orange, Cleopatra mandarin and Rusk citrange. There was no difference in the water tolerance of the seedlings in the limed soil after 3 weeks of continual flooding. After 6 weeks of continual flooding the order of decreasing water tolerance of the seedlings was: Troyer citrange, Carrizo citrange, sour orange, Rough lemon, sweet orange, Cleopatra mandarin and Rusk citrange. In the flooded cans where there was more than one seedling, the order of decreasing water tolerance during the summer months was: Rough lemon, Cleopatra mandarin, sour orange, and sweet orange.

Sour orange rootstock has been classified as being more resistant to water injury than either sweet

orange or Rough lemon rootstocks. This was attributed to the more shallow rooting tendency of the sour orange rootstock by Rhoads (72). Others (45,46) have attributed these difference to the greater resistance of sour orange rootstock to root-rotting fungi. If the soil environment is conducive to growing vigorous plants with good root systems the difference between the water tolerances of the various rootstocks would be due to the length of time the waterlogged condition was present throughout the soil profile in which the roots were concentrated. The rate and degree to which new roots are formed under prolonged waterlogging is also a contributing factor in the water tolerances of citrus seedlings.

IV. DEMONSTRATION AND EVALUATION OF TOXINS AS A FACTOR ASSOCIATED WITH WATER DAMAGE

Very little is known about why some plants may become injured so quickly when the soil in which they are grown becomes flooded. Lack of oxygen, increase of carbon dioxide, and the production of toxic substances have been suggested as the causes of flood injury. Toxins were considered to be a possible causal agent and therefore preliminary experiments were designed to determine whether a soil kept under stagnant conditions would produce toxic substances which would be detrimental to citrus plants. It was further realized that this would not be the same condition as found in citrus grove soil when water-logged. Therefore, fresh citrus roots were incorporated in soil and all of it submerged in water. It was found that citrus seedlings wilted in the soil water where citrus roots had been but remained unwilted in the soil water where there had been no citrus roots.

The purpose of these experiments was to investigate the production and properties of a toxin in citrus root solutions which causes seedlings to wilt. The effects of temperature, pH, and microorganisms on the

production of the toxin were investigated. The influence on citrus seedlings of water extracts from stagnant water-logged soils with and without citrus roots and the subsequent effect on healthy seedlings of planting them in these soils and reflooding were determined.

Methods and Results

Various methods were used to study the properties of the toxin in the root water. Filtering, activated carbon, heat, vacuum and atmospheric distillation, exchange resins, varied pH values and nutrient precipitations, alcohol and acetone precipitation, and ether extractions were all employed to remove the toxin from the root solution. Paper chromatography and fluorimetry were also used to gain further information on the toxin.

Demonstration of the production of a toxin.

Citrus feeder roots and small lateral roots from healthy trees were selected for incubation in water in sealed glass jars. This incubated root water was tested on Rough lemon, sour orange, sweet orange, and Cleopatra mandarin seedlings for the presence of toxic substances which induce wilting. The jars were incubated at different temperatures, the pH was adjusted prior to incubating, different quantities of roots were incubated in soil and water, and citrus roots from trees on Cleopatra mandarin, Rough lemon, sour orange and sweet

orange stock were inoubated.

Experiment I. Effeot of quantity of roots.- Two pint jars each with 10 grams of fresh citrus feeder roots were filled with water. Six Rough lemon seedlings were placed in 1 jar and 6 sour orange seedlings were placed in the other. Two pint jars filled with deionized water had 6 Rough lemon seedlings in one and 6 sour orange seedlings in the other. These seedlings were held upright by a oircular waxed perforated cardboard and a metal ring cap, and the jars were placed in the greenhouse. All of the seedlings in the jars with the added citrus roots were found to have wilted after 2 weeks. The seedlings in the jars with no additional roots remained turgid.

Six one-quart Mason jars were partly filled with 600 grams of Lakeland fine sand from an old citrus grove. To eaoh of these jars was added respeotively 0, 1, 5, 10, 25, and 35 grams of fresh citrus feeder roots and these wsre incorporated in the soil. Another 6 one-quart Mason jars were partly filled with 600 grams of Leon fine sand from a pasture with native cover. To each of these jars was also added respectively 0, 1, 5, 10, 25 and 35 grams of fresh citrus feeder roots and these were incorporated in the soil. All of these jars were filled with deionized water, sealed and incubated

in the laboratory at room temperature for 2 weeks. A one-quart jar with 10 grams of citrus roots was filled with water only and incubated alongside the other jars. Each jar was shaken at the end of the incubated period and 8 citrus seedlings (2 each of the four varieties of citrus seedlings) were placed in each jar. Another 8 seedlings were supported in a quart jar of deionized water as control. The jars were covered with aluminum foil and placed in the greenhouse.

After 7 days only the 2 Rough lemon seedlings in the jar which had the Lakeland soil and 25 grams of citrus roots were wilted. However, all of the seedlings in the jars which had the Leon soil with 10, 25, and 35 grams of citrus roots were wilted after 7 days. Those in the jar with roots and water without soil were also wilted. After 2 months the seedlings in the jars with both types of soil containing no roots, and in the jars with Lakeland soil and 1, 5, and 10 grams of citrus roots were still turgid and healthy except for the sour orange seedlings, which developed yellow-veined leaves. Less than one-fourth of the water remained in these jars after a two-month growing period. The seedlings in the remaining jars were all wilted at the end of the two-month period with approximately 90 per cent of the water

still in each jar. The seedlings in the deionized water remained turgid for 2 months with only yellow-vein symptoms on 1 of the sour orange seedlings.

Experiment II. Effect of parts and kinds of citrus roots.- One hundred grams each of fresh root cortex (from xylem outward), root xylem (wood), and feeder roots from older trees of Rough lemon, sweet orange, sour orange and Cleopatra mandarin rootstocks were incubated separately in sealed gallon jars filled with water for 2 weeks in the greenhouse. Six each of Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings were placed in 480-ml aliquots of root solutions from each of the incubated jars. For controls, 6 each of Rough lemon, sour orange and sweet orange seedlings were placed in 480-ml quantities of deionized water.

Twenty-five grams of small lateral and feeder roots from trees which had been subjected to high water and were in a wilted condition were incubated in 480 ml of deionized water. Both the feeder and lateral roots were badly sloughed. The water table at the time the wilted trees were examined was 4 feet below the surface. Twenty-five grams of roots from an apparently healthy tree in close proximity were also incubated in 480 ml of deionized water. After 2 weeks of incubation 3 sour orange seedlings were placed in each root solution.

After 7 days all of the seedlings in all of the root solutions were wilted, and they were desiccated after 10 days. The seedlings in the deionized water remained green and turgid. The stems of the sour orange seedlings in the root solution from the apparent water-damaged roots and non-water-damaged roots after 7 days were bleached in addition to being wilted.

Experiment III. Effect of temperature.- One hundred grams of feeder roots from old citrus trees were incubated in sealed gallon jars filled with water at 32° F., 40° F., 60° F., 72° F., 80° F., and greenhouse temperature for 7 and 14 days. Also, 2 one-gallon jars each with 100 grams of feeder roots were filled with water, and placed in the greenhouse, and compressed air was bubbled continuously into one of them for 7 days and into the other for 14 days. Six each of Rough lemon, sour orange, and sweet orange seedlings were placed in 480-ml aliquots of the root solution from each of the incubated jars and in 480 ml of deionized water.

The influence of temperatures at which citrus roots were incubated on the wilting of citrus seedlings are presented in Tables 5 and 6. The greatest difference between the 7-day and 14-day incubations was the subsequent wilting effect of the aerated solutions on

TABLE 5.- The influence of temperatures at which citrus roots were incubated for seven days on the wilting of citrus seedlings.

Incubation temperature	Seedlings[a]	Number wilted after days in solution 7	16
60° F.	RL	3	4
	SO	4	5
	SwO	6	3
72° F.	RL	6	6
	SO	6	5
	SwO	6	5
80° F.	RL	6	6
	SO	6	6
	SwO	6	6
Greenhouse	RL	2	6
	SO	6	6
	SwO	6	6
Greenhouse ↑ air	RL	0	0
	SO	0	0
	SwO	0	0
Deionized water	RL	0	0
	SO	0	0
	SwO	0	0

(a) Six seedlings per variety per 480-ml aliquots of solution.

TABLE 6.- The influence of temperatures at which citrus roots were incubated for fourteen days on the wilting of citrus seedlings.

Incubation temperature	Seedlings[a]	Number wilted after days in solution	
		4	11
60° F.	RL	4	6
	SO	6	6
	SwO	6	6
72° F.	RL	6	6
	SO	5	5
	SwO	6	6
80° F.	RL	4	6
	SO	6	6
	SwO	6	6
Greenhouse	RL	6	6
	SO	6	6
	SwO	6	6
Greenhouse + air	RL	1	6
	SO	4	6
	SwO	6	6
Deionized water	RL	0	0
	SO	0	0
	SwO	0	0

(a) Six seedlings per variety per 480 ml of solution.

the citrus seedlings. The seedlings in the 14-day incubated solution were all wilted on the eleventh day whereas none of the seedlings had wilted in the 7-day incubated solution.

No seedlings were wilted after 1 month in the root solutions which had been incubated either at 32° F. or at 40° F. Following the removal of the aliquots from the jar which had been incubated at 40° F., the jar was resealed and incubated for an additional 2 weeks in the greenhouse. Four Rough lemon seedlings were then placed in 480 ml of the root solution. All of these Rough lemon seedlings were wilted after 7 days in the root solution.

Experiment IV. Effect of pH.- Forty grams of dried citrus feeder roots were incubated in sealed gallon jars filled with water for 11 days in the greenhouse after the pH was adjusted to 4.0, 6.0 and 7.5 with hydrochloric acid or sodium hydroxide. One jar was incubated without any pH adjustment and its initial pH was 5.6. All pH measurements were made using a glass electrode and a Beckman pH meter. The pH of the solution in each jar, and of the solutions in the jars in which the citrus seedlings had been for 11 days, was measured before and after incubation. Five each of Rough lemon, sour orange, sweet orange and Cleopatra mandarin

seedlings were placed in 480-ml aliquots of the incubated root solutions.

The influence of pH of the solution in which citrue roote were incubated on the wilting of citrue seedlinge ie presented in Table 7. After 5 days, of the citrus seedlings in the eolutione which had an initial pH of 4.0, lese than one-half of the eeedlings were wilted. Thoee eeedlings that were wilted were eour orange, sweet orange and Cleopatra mandarin.

The changes in tho pH of the incubated solution and of the solutions in which the different seedlings had been for 11 days are recorded in Table 8. The pH after incubation of the solutions with initial pH valuee of 5.6, 6.0, and 7.5 was lowered to 4.8. The solution with an initial pH of 4.0 had a pH of 5.1 following incubation. The pH of the solutions in which seedlinge had been for 11 days ranged from 5.1 to 7.5. The pH of the solutione which had an initial pH of 4.0 wae higher after the seedlinge had been in them than that of any other eolutione.

Experiment V (a). Effect of microorganiems.- Twenty-five grams of oitrue feeder roote from old citrue treee were incubated in eealed one-quart jare with 100 grams of Leon topeoil (0-7"), 100 grams of Leon eubeoil

TABLE 7.- The influence of pH of the solution in which citrus roots were incubated on the wilting of citrus seedlings.

Initial pH[a]	Seedlings[b]	Number wilted after days in solution			
		2	3	4	5
4.0	RL	0	2	0	0
	SO	2	2	2	2
	SwO	2	3	3	2
	Cleo	2	4	3	3
5.6	RL	4	4	5	5
	SO	4	5	5	5
	SwO	4	5	5	5
	Cleo	5	3	5	5
6.0	RL	5	5	5	5
	SO	4	5	5	5
	SwO	5	5	5	5
	Cleo	5	5	5	5
7.5	RL	4	5	5	5
	SO	4	4	5	5
	SwO	5	5	5	5
	Cleo	5	5	5	5

(a) Incubated for 11 days.

(b) Five seedlings per variety per 480-ml aliquots of solution.

TABLE 8.- The pH changes of the solutions following incubation and following the wilting of the seedlings.

Initial pH	pH after 2 weeks incubation	pH after 11 days with seedlings RL	SO	SwO	Cleo
4.0	5.1	7.2	7.3	7.5	7.4
5.6	4.8	6.7	6.6	6.7	6.7
6.0	4.8	6.7	6.2	6.3	6.5
7.0	4.8	6.7	7.0	6.1	6.8

(7-17") and no soil. The jars were all filled with water. After 2 days of incubation in the greenhouse 1 ml of a 1:1000 dilution of the water from each jar was placed on petri dishes which contained 4 agar media: potato dextrose, pH 3.0 (20); dextrose, pH 7.3 (20); orange serum, pH 6.8 (20); and mycological. The mycological agar medium had the following composition (grams): glucose, 10; peptone, 5; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.5; agar, 30 in 1000 ml distilled water, and the pH was adjusted with H_2SO_4 to 4.0.

Duplicate petri dishes of each agar medium were inoculated. One-half of the petri dishes were incubated at 32° C. and the other half were incubated at room temperature in the laboratory desk for 1 week. The

dishes which had prolific growth within 2 days were removed from their respective incubators and stored in the 0° C. room.

Twenty-five grams of citrus roots in 1000 ml of water were sterilized in an autoclave at 15 pounds pressure for 30 minutes in each of 9 two-liter flasks. These flasks were checked for sterility by streaking orange-serum plates with water from each flask using a sterile loop.

The flasks with the autoclave-sterilized roots were inoculated with the growth on the different agar media in the following manner:

Flask No.	Agar medium	Source of inoculum
F-1	Dextrose	Roots and subsoil
F-2	Orange serum	Roots and subsoil
F-3	Mycological	Roots and subsoil
F-4	Mycological	Roots only
F-5	Orange serum	Roots only
F-6	Potato dextrose	Roots only
F-7	Potato dextrose	Roots and topsoil
F-8	Potato dextrose	Roots and topsoil
F-9	---	---

Two milliliters of sterile water were poured on each plate and a sterile loop was used to loosen the growth. Each mixture was poured into a bottle with sterile water. Cell counts were made on the solution using the direct microscopic method. The quantity of solution added to each flask contained approximately 100,000 cells. These flasks were incubated in the laboratory desks at room

temperature for 10 days after each was inoculated. Also 1 flask containing 25 grams of unsterilized roots in 1000 ml of water was incubated for 10 days.

All flasks which had been autoclaved were sterile according to the streaked orange-serum dishes.

The bacterial colonies on the inoculated dextrose and orange-serum media were too numerous to count after 24 hours in the 32° C. incubator. However, the media to which water had been added from the jar with citrus roots and topsoil had a greater number than those plates to which water was added from the jar with citrus roots and subsoil. The smallest number of colonies on these media was from the water in the jar that contained just citrus roots.

There were colonies of fungi in all mycological agar dishes after 72 hours. The different fungal colonies were grown together. There were from 1 to 6 colonies of fungi on the potato-dextrose-agar plates after 3 days of incubation under both incubating conditions.

Following the incubation period of the inoculated flasks, seedlings were placed in two 250-ml aliquots of solution from each flask. One aliquot contained 5 sweet orange and 3 Rough lemon seedlings and the second aliquot contained 3 Rough lemon and

5 sour orange seedlings. These containers were placed in the greenhouse.

None of ths seedlings in any of the solutions was wilted after 7 days. These seedlings remained green for 2 weeks at which time less than one-half of the solution remained in the containers.

Experiment V (b). Effect of surface washing.- Citrus roots were washed with an intense spray of water. Fifty grams of these roots wers placed in one-liter Erlenmeyer flasks. Three flasks were treated for 3, 5, and 8 minutes with a 3.5 per cent sodium hypochlorite solution to which 11 ml of glacial acetic acid per liter had been added. Two flasks were treated with a 0.25 per oent sodium hypochlorite solution to whioh 3 ml of glacial acetic acid per liter had bean added. One flask which was untreated and filled with water served as a check. The roots were rinsed four times with sterile water by covering the roots and dscanting (after ths hypochlorite solutions were poured off). After the roote were rinsed the flasks were filled with sterile water and sealed with a sterile rubber stopper. Thess flasks were incubated in the laboratory desks. Duplicate petri dishes with orangs serum and potato dextrose agar were streaked with water from each flask after 3 days of incubation. One set was incubated at

32° C. and the other set at room temperature.

After the flasks were incubated for 14 days, 3 sweet orange seedlings were placed in 250-ml aliquots from each flask.

Thirty grams of washed citrus roots were placed in 1000 ml Erlenmeyer flasks. Two of these flasks were filled with a 800 ppm Roccal solution (active ingredient: alkyl benzyl ammonium chloride) for 30 minutes and 2 flasks for 60 minutes. This solution was decanted and the flasks were filled for 10 minutes with a 0.25 per cent sodium hypochlorite solution to which 3 ml of glacial acetic acid per liter had been added. The roots were rinsed three times with sterile water and then each flask was filled with sterile water and sealed with a sterile rubber stopper. One flask with untreated roots was filled with sterile water and sealed with a rubber stopper. The stoppers in each flask were covered with aluminum foil and the flasks were incubated in the greenhouse. Duplicate petri dishes with orange-serum and potato-dextrose agar were streaked with water from each flask after 3 days of incubation. One set of the dishes was incubated at 32° C. and the other set at room temperature in laboratory desks.

After the roots were incubated for 2 weeks, 6 sour orange seedlings were placed in 480-ml aliquots of the root solution from each flask.

None of these flasks was sterile. On the orange serum medium which had been streaked with the solution from the flask with untreated roots both bacteria and molds were present. Only bacterial colonies appeared on the orange serum medium which had been streaked with water from the Roccal- and hypochlorite-treated citrus roots. Fungal colonies appeared only on the potato dextrose which had been streaked with water from the hypochlorite-treated and untreated roots. The Roccal solution apparently inhibited the fungal colonies.

After 7 days in the root solutions all of the sweet orange seedlings were wilted. The sweet orange seedlings in the solution from the flask with the untreated roots were desiccated after 5 days. More than one-half of the sour orange seedlings in the solution from the flask with the untreated roots were desiccated after 5 days. More than one-half of the sour orange seedlings in each solution were wilted after 7 days. All of them were wilted after 14 days.

Experiment VI. Effect of stagnant water from flooded Leon soil.- One-gallon metal cans containing

Leon subsoil and topsoil from the native pasture were flooded with deionized water in the greenhouse. One group of cans had Rough lemon seedlings growing in the topsoil. A second group of cans had Rough lemon seedlings growing in the subsoil. A third group had 70 grams of roots from old citrus trees incorporated in each gallon of subsoil (prior to flooding). A fourth group of cans contained the virgin subsoil. After 2 weeks of flooding the seedlings were wilted and desiccated. The water from each group of cans was removed with the aid of a water aspirator and a piece of glass tubing with a fine mesh wire covering the end. The soil water from each group of cans was passed through chopped filter paper using suction. The filtered soil water was a clear tan solution. Sweet orange seedlings were put in each filtered soil extract. There was one seedling per 50 ml of solution.

Rough lemon and sweet orange seedlings were planted in these cans after the water was extracted, and the cans were reflooded with deionized water.

The sweet orange seedlings in the soil water extract from the cans which had the wilted seedlings and the incorporated citrus roots were all wilted after 7 days. However, the sweet orange seedlings in the soil water extract from the virgin soil were not wilted in

7 days nor were they wilted after 3 weeks.

The Rough lemon and sweet orange seedlings which were planted in the previously flooded soil (in which Rough lemon seedlings had wilted and citrus roots were incorporated) and were reflooded after planting were all wilted after 7 days. The sweet orange seedlings in virgin soil and treated similarly remained turgid for 1 month in spite of the continued flooded condition.

Evaluation of a toxin in root solutions.

Wilting test.- Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings were used to determine the presence of the toxin which causes seedlings to wilt. The same kind of seedlings in deionized water were used as a check and in some tests the original solution was also used as a comparison check. After 7 days the seedlings were rated as wilted or not wilted.

Preparation of solutions containing the toxin for evaluation.- One hundred grams of freshly dug citrus feeder roots from old trees were washed thoroughly with water and placed in gallon glass jars which were filled with deionized water, sealed, and incubated from 10 to 14 days in the greenhouse. The resulting solution was first passed through a Whatman No. 41 filter paper to

remove large particles and then through a Seitz filter pad.

Activated carbon adsorption.- Activated carbon was added to the root solution (1 per cent w/v). One volume was boiled vigorously for 5 minutes and the other volume remained at room temperature for 30 minutes. Each volume was filtered to remove the activated carbon. Six seedlings were put in 480 ml of the filtrate of both the heated and the non-heated solution. All Rough lemon, sour orange, sweet orange and Cleopatra mandarin seedlings wilted the same as those in the solution to which no activated carbon was added. This was repeated four different times with similar results.

Exchange resin adsorption.- One hundred millilitres of root solution were passed through 30 ml of cation exchange resin (Amberlite IR-120 in H^+ form). An equal volume of solution was passed through 30 ml of anion exchange resin (Amberlite IRA-400 in OH^- form). Before seedlings were placed in the filtrate the pH was adjusted to the range of 5.0-6.0 with sodium hydroxide or sulfuric acid solutions. One sweet orange seedling per 50 ml of filtrate was used to test for the presence of the toxin.

All seedlings wilted in the filtrate from both the cation and anion resin and in the original solution. Although the filtrate from the cation exchange resin caused the seedlings to wilt sooner than that from the anion exchange resin, the seedlings in water and a nutrient solution remained green and turgid. The sweet orange seedlings in the cation exchange filtrate of root solutions (400 grams of old citrus and young seedling roots per gallon) were bleached after 7 days but the seedlings in the anion exchange filtrate and the original solution were not bleached.

Nutrients and pH.- Nutrients were added to aliquots of the root solution (per liter: 0.5 M $Ca(NO_3)_2$, 9 ml, and 0.5 M KH_2PO_4, 4.6 ml). The pH of the solutions with and without the added nutrients was adjusted with $Ca(OH)_2$, NaOH, HCl, or H_2SO_4. Eight citrus seedlings were used to test the presence of the toxin in 480 ml of the resulting solutions. The influence of nutrients and the pH change of the solution on the subsequent wilting effect of the treated solution on citrus seedlings is presented in Table 9. A precipitate was formed in the solutions with $Ca(NO_3)_2$ and KH_2PO_4 when the pH was adjusted above 7.0 with NaOH or $Ca(OH)_2$. No precipitate occurred when the pH of the

TABLE 9.- The influence of nutrients and pH of the root solution on the subsequent wilting effect of the treated solution on citrus seedlings.

Materials[a] added	Initial pH	Number of seedlings wilted[b]
None	4.8	8
$Ca(NO_3)_2$ + KH_2PO_4[c]	4.8	0
$Ca(NO_3)_2$, KH_2PO_4	4.9	8
$Ca(NO_3)_2$, KH_2PO_4 and $Ca(OH)_2$	7.6	5
$Ca(NO_3)_2$, KH_2PO_4 and NaOH	7.4	5
H_2SO_4	4.0	8
HCl	4.0	8
NaOH	6.0	6
NaOH	7.5	1
$Ca(OH)_2$	7.7	0

(a) Per liter: 0.5 M $Ca(NO_3)_2$, 9 ml; 0.5 M KH_2PO_4, 4.6 ml; $Ca(OH)_2$, saturated solution; HCl, 1:1; NaOH, 5 N; H_2SO_4, 1:10.

(b) Out of a total of 8 seedlings per 480 ml and the number wilted after 7 days.

(c) No root solution.

solutions without $Ca(NO_3)_2$ and KH_2PO_4 was adjusted above 7.0, nor was there any precipitate formed when either $Ca(NO_3)_2$ or KH_2PO_4 was present alone and the pH adjusted to 7.0.

The precipitate was calcium phosphate and as it was formed possibly some of the toxin was precipitated and thus reduced the concentration of the toxin. The base changed the toxin to a less toxic form where there were no ions present to precipitate. Two acids and two bases were used to compare the cationic and anionic effects on the seedlings, if any. Apparently there was no effect because of the presence of either, but the pH change had a striking influence on the toxin.

The seedlings in the nutrient solution made with deionized water grew with no evidence of wilting for a period of 3 weeks in spite of the lack of aeration.

Thermostability.- Five hundred milliliters of root solution were refluxed vigorously for 15 minutes in an Erlenmeyer flask, cooled, and transferred to jars. Six sour orange seedlings were inserted, and an equal number of seedlings were placed in 500 ml of unboiled root water. All seedlings wilted.

Two thousand milliliters of root solution were autoclaved at 15 pounds pressure for 30 minutes. The

solution was cooled and was divided into four equal portions. Into each portion 6 Rough lemon, 6 sour orange, 6 sweet orange and 6 Cleopatra mandarin seedlings were inserted respectively. All seedlings wilted.

Volatility.- Five hundred milliliters of root solution were evaporated to dryness with heat. The residue was mixed in 500 ml of water and 6 sour orange seedlings inserted. These seedlings remained green and turgid for 2 months at which time new roots had formed.

Another 500 ml of root solution were reduced to approximately 5 ml on a hot plate and made up to a 500-ml volume. Six sour orange seedlings were inserted for 2 months. The seedlings remained green and turgid and formed new roots.

A volume of root solution was concentrated under vacuum at approximately 45° C. using a Rotary Flask Evaporator. One liter was reduced to 21 ml. The distillate was collected in the condenser. The evaporator was rinsed with 29 ml of water and the concentrate was centrifuged to separate the precipitate which had formed as the volume was reduced. The precipitate was dissolved in 100 ml of water and the supernatant solution was also made to a volume of 100 ml with water. One Rough lemon seedling was put in 50-ml aliquots of

each solution. The seedlings in the precipitate solution did not wilt, but those in the supernatant solution wilted. Subsequently tests with sour orange and sweet orange seedlings in the supernatant solution also proved the existence of wilt-inducing toxic properties. Four Rough lemon seedlings were placed in a 300-ml aliquot of the distillate in the condenser of the evaporating unit and 2 in the last 150 ml of distillate. All seedlings wilted.

Five hundred milliliters of root solution were evaporated to dryness under vacuum. The residue was mixed in 120 ml of water and sour orange seedlings were inserted into 50-ml aliquots. No seedlings wilted.

An attempt was made to collect 100-ml aliquots of 500 ml of root solution (pH 4.8) as it was evaporated under vacuum. One sweet orange seedling was inserted into each aliquot. All of these seedlings wilted. In addition, the seedlings in the last 2 aliquots were desiccated on the seventh day. This was repeated with another 500 ml of root solution which had the pH adjusted to 9.0 with NaOH before it was evaporated under vacuum. One sweet orange seedling was inserted into each aliquot. Only the seedling in the last aliquot wilted.

Fifty milliliter-aliquots of root solution (acid)

were evaporated to dryness at room temperature with the aid of an electric fan, and other 50-ml aliquots of root solution, to which a NaOH solution was added to make the solution basic, were also evaporatsd under a similar condition. Each dried residue was redissolved in 50 ml of watsr and the pH of the solutions was adjusted to 5.3 with H_2SO_4. One swest orangs seedling was put into each 50-ml volume. Only the seedlings in the solutions of the residue from the basio root solution wilted.

Distillation.- Five hundred milliliters of each of 3 root extraots and the water from flooded soils wers distilled separately at atmospherio prssure. The root solutions which were distilled were from jars with 100 and 400 grams of fresh feeder roots per gallon. The water was extracted from Leon topsoil and subsoil in which Rough lemon seedlings were killed after the soil was flooded, from flooded subsoil to which fresh citrus feeder roots were added prior to flooding, and from flooded virgin subsoil. The soil was in gallon cans and flooded for 2 weeks in the greenhouse before the water was extracted with a fine-mesh-wire-covered glass tube and an aspirator. The metal cans rsmained bright. The extracted soil water was filtered through chopped Whatman No. 1 filter paper in a Büchner funnel. Ten 50-ml fractions of each were collected in each

dietillation. The reeidue in each of the dietillation flasks wae reeuepended in 500 ml of water. One eweet orange seedling per 50-ml distillate fraotion, root solution, and extraoted eoil water was ueed to teet for the preeence of a toxin which producee wilting.

The responee of eweet orange eeedlinge in the root eolutions and the distillate fractione is preeented in Table 10. After 7 days only 3 eeedlinge in the dietillate fraotions from the feeder root solution (100 grams per gallon) remained turgid. Theee were wilted after 14 days. The eeedling in the tenth dietillate fraotion of the feeder root eolution (400 grame per gallon) began to turn brown after 48 houre and was white by the seventh day as shown in Figure 22. Figure 23 illuetrates the bleaching effect that the distillate fraotions from the lateral root eolution had on sweet orange eeedlinge. The eeedlinge in thie root eolution were wilted in 48 houre with bleached etems and were desiocated by the eeventh day.

The reeponse of eweet orange eeedlinge in the eoil water extracte and the dietilled fractions are preeented in Table 11. The eeedlinge in the water extraot from the soil in which Rough lemon roote were preeent either with the top or without and had been

TABLE 10.- Response of single sweet orange seedlings in incubated root solutions and the distilled fractions therefrom.

Fractions[a]	Solutions A[c]		B		C	
	Days 7	14	7	14	7	14
1	1[d]	2	2	2	4	4
2	2	2	2	2	3	4
3	2	2	2	2	3	4
4	2	2	2	2	4	4
5	2	2	2	2	3	4
6	2	2	2	2	2	2
7	2	2	2	2	4	4
8	1	2	2	2	4	4
9	2	2	2	2	3	4
10	1	2	3	3	3	4
OS[b]	2	2	2	2	4	4

(a) The plants did not wilt in the resuspended residue solution, water or nutrient solution.

(b) OS- original solution.

(c) A- 100 grams feeder roots per gallon.
B- 400 grams feeder roots per gallon.
C- 400 grams lateral roots per gallon.

(d) 1- turgid.
2- wilted.
3- turgid and partly bleached.
4- wilted and partly bleached.

Figure 22.- Response of sweet orange seedlings after twenty days in a citrus feeder root solution (400 grams per gallon), in distilled fractions, the resuspended residue and water. Left to right: resuspended residue, root solution, fifth fraction, tenth fraction and water.

Figure 23.- Response of sweet orange seedlings in nine of the ten distilled fractions of a lateral root solution (400 grams per gallon) and in water. Left to right: fractions one through ten (fraction two is omitted) and water.

TABLE 11.- Response of single sweet orange seedlings in soil water extracts and in the distilled fractions therefrom after seven and fourteen days.

Fractions[a]	Solutions							
	T[c]		S		R		N	
	Days							
	7	14	7	14	7	14	7	14
1	3	4	2	2	1	1	1	1
2	3	3	2	2	1	1	1	1
3	2	4	2	2	1	2	1	1
4	3	3	1	2	2	2	1	1
5	3	4	1	1	1	2	1	1
6	3	4	2	2	2	2	1	1
7	4	4	1	2	2	2	1	1
8	4	4	1	1	1	2	1	1
9	2	2	2	2	1	2	1	1
10	3	3	3	3	1	2	1	1
OS[b]	2	2	2	2	2	2	1	1

(a) The plants did not wilt in the resuspended residue solution.

(b) OS- original solution.

(c) T- topsoil- Rough lemon seedling.
S- subsoil- Rough lemon seedling.
R- subsoil- added roots.
N- subsoil.

(d) 1- turgid.
2- wilted.
3- turgid and partly bleached.
4- wilted and partly bleached.

flooded for 2 weeks were wilted in 7 days. No seedlings were wilted in the water extract from the flooded soil where there had been no citrus roots or in its distillate fractions. Figures 24 and 25 illustrate the comparative response of sweet orange seedlings in two distillate fractions and the soil water extracts from the topsoil and subsoil in which Rough lemon seedlings had been damaged to those in water. The seedlings in the tenth fractions were almost completely bleached. The seedlings in the fourth distillate fractions of the soil water extract from the topsoil with the Rough lemon seedling had bleached stems and petioles whereas there was no bleaching on the seedlings in the same fractions from the soil water extract of the subsoil with the Rough lemon seedling.

Ether extraction.- Ethyl ether was used to determine whether the toxin could be removed from root solutions. The outline of the procedure used in the ether extractions is presented in Figure 26. Two successive ether extracts were made of acidified solutions (pH 2.1) and two of basic solutions (pH 9.5). The ether extracts were evaporated in a fume hood after the addition of a little distilled water. The pH of the resulting root solutions and the water after the ether was fanned off was adjusted to pH 5.1 with

Figure 24.- Response of sweet orange seedlings in distilled fractions and in original soil water extract from flooded Leon topsoil in which Rough lemon seedlings had wilted. Left to right: fourth fraction, tenth fraction, soil water extract, deionized water.

Figure 25.- Response of sweet orange seedlings in distilled fractions and in original soil water extract from flooded Leon subsoil in which Rough lemon seedlings had wilted. Left to right: fourth fraction, tenth fraction, soil water extract, deionized water.

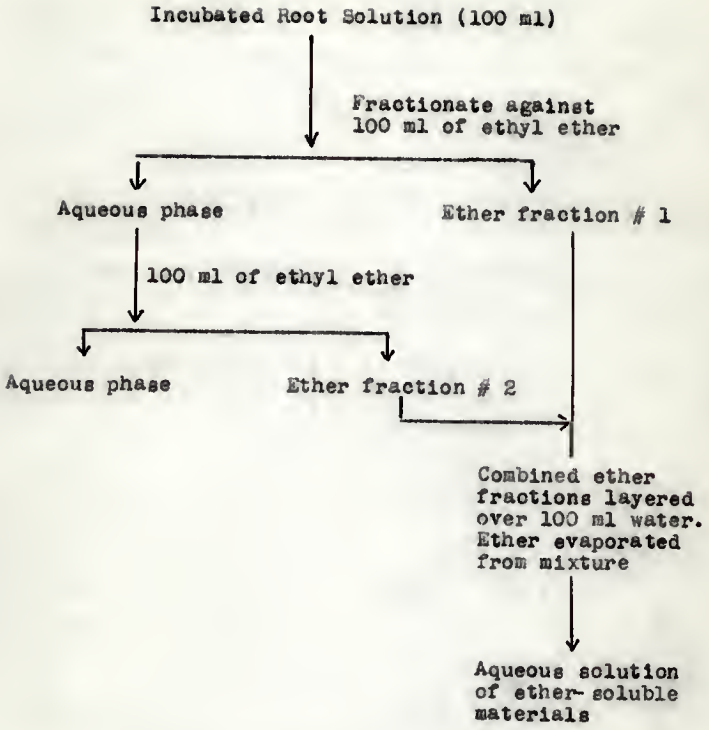

Figure 26.- Procedure used in the separation of the toxin from the root solution with ethyl ether.

H_2SO_4 or KOH. Sweet orange seedlings were placed in 50-ml aliquots. Two root solutions were used: A, 100 grams feeder roots per gallon of water, and B, 400 grams lateral roots per gallon of water.

The response of the sweet orange seedlings in the resulting ether extracted solutions is presented in Table 12. Two ether extractions of the acid root solution A partitioned the toxin from the aqueous medium, and when the ether was layered over water and fanned off the toxin was left dissolved in the water, as was indicated by the wilting of the sweet orange seedlings. The seedlings in the acid root solution B after ether extraction wilted, as well as the seedlings in the water after the ether from the acid water was fanned off. Two ether extractions were not sufficient to remove all of the toxin from this acid solution, which was much more concentrated than solution A. However, a sufficient quantity was extracted and reabsorbed in the water to cause the seedlings to wilt.

Acetone and ethanol precipitates.- Attempts were made to isolate the toxin by precipitation. A modification of the method of Feldman et al. (23) was used. Acetone and ethanol were added to root solutions in a 3:1 (solvent: root solution) proportion. Following the settling of the precipitate

TABLE 12.- Response of sweet orange seedlings in the resulting root solutions from ether extraction after seven days.

Solution[(a)]	Plant response
A	
Acid solution after ether extraction	turgid
Water solution of ether soluble fraction	wilted
Basic solution after ether extraction	wilted
Water solution of ether soluble fraction	turgid
Original solution	wilted
B	
Acid solution after ether extraction	wilted
Water solution of ether soluble fraction	wilted
Basic solution after ether extraction	wilted
Water solution of ether soluble fraction	turgid
Original solution	wilted

(a) A- 100 grams feeder roots per gallon.
B- 400 grams lateral roots per gallon.

the mixture was centrifuged. The precipitate was redissolved in water making a concentration approximately five times that of the solution from which the precipitate came. The acetone or ethanol was evaporated on a hot plate. Seedlings were placed in the precipitate solution and in the filtrate after the acetone and ethanol had been removed by evaporation.

None of the seedlings in the precipitate solution wilted, but all of them wilted in the filtrate. The test was repeated six times with similar results.

Paper chromatography.- Paper chromatography was used in an attempt to isolate the toxin. Descending chromatography was employed in a Chromatocab and Chromatographic jars. The root solution was applied 3 inches from one end of Whatman No. 1 filter paper. Two sizes of paper were used: 18 x 22½ inches and 7 x 22½ inches. Two solvents used were butanol: acetic acid: water (4:1:5) and distilled water. The papers to which 50 ml of root solution had been added were sprayed for organic acids, polyphenols, reducing substances and amino acids. The reagents used were bromocresol green, diazotized sulfanilic acid, silver nitrate and ninhydrin, respectively, and were prepared as outlined by Block, Durrum and Zweig (6). An ultraviolet lamp with a wavelength of 367 mμ was used to

detect any fluoreecent spots.

Ten milliliters of root eolution were etreaked on eaoh of 6 large sheete of paper in 1 ml units. Water wae the moving solvent. The different fluoreecent areas were cut in etripe and each strip, fluoreecent and non-fluorescent, was eluted with approximately 10 ml of water until no fluorseoence remained on the paper. The eluatee from similar stripe were mixed togethsr and 1 sweet orange seedling wae inserted in each eluted fraction.

No organic acide, reducing eubstancee, polyphenols or amino acide were detectable. There were at lsaet 5 definite fluorescent epots which eeparated with both solvente from all citrus root solutione employed, feeder or lateral. None of these areas when elutsd caueed the eeedlings to wilt. However, the eesdlinge in the non-eeparated root solution wilted. A strong offeneive odor was detected when ths paper was drying aftsr sach streaking.

Spectroscopy.- Maximum absorption peaks in the ultraviolst rsgion wers detsrmined for ths eluted fraotions from the paper, using a meroury lamp and the Beckman DU Spectrophotometer. Also maximum abeorption peake wers determinsd on ssveral of the dietillate fractione from both the root solutions and the soil water

extracts. The percentage of transmission of fluorescence was determined on the distillate fractions of root solutions and soil water extracts at wavelength 365 mμ. Deionized water was used as the comparative standard.

The 7 peaks in the ultraviolet spectral curve were at 296.5, 302, 313, 334, 365, 402 and 436 mμ. The most intense peak was at 365 mμ.

The first distilled fraction of the soil water extracts in which there had been citrus roots had a higher percentage of transmission than fractions two through nine (see Table 13). The tenth fraction had the most intense fluorescence, which was two to three times that of fraction number one. The distillate fractions of the soil water extract from the soil in which no citrus roots had been had a fairly constant percentage of transmission ranging from 8-12 with the exception of fraction number one. The distillate fractions from the root solutions showed a steady increase in fluorescent intensity from fraction one to fraction ten, as measured by the transmission percentages.

TABLE 13.- Percentage transmission[a] of distilled fractions of soil water extracts and root solutions at 365 mμ.

Fractions[b]	Solutions[c]						
	T	S	R	N	A	B	C
1	23	20	28	28	18	23	17
2	9	10	10	12	22	20	16
3	10	10	10	12	20	28	19
4	12	10	12	12	25	27	21
5	12	10	14	8	25	33	21
6	12	10	14	7	29	33	25
7	12	19	16	9	31	36	30
8	17	24	17	9	40	42	36
9	21	31	25	8	52	54	45
10	57	60	50	8	63	64	47

(a) Slit width used for soil water extracts was 0.7 mm and for root solutions was 0.35 mm.

(b) Five hundred milliliters distilled into 50-ml fractions.

(c) T- topsoil with Rough lemon seedling.
S- subsoil with Rough lemon seedling.
R- subsoil with added citrus roots.
N- subsoil.
A- 100 grams feeder roots per gallon of water.
B- 400 grams feeder roots per gallon of water.
C- 400 grams lateral roots per gallon of water.

Discussion

A preliminary experiment demonstrated that water from containers in which citrus roots had been incubated with and without soil caused citrus seedlings to wilt rather rapidly. Further experiments with incubated citrus roots in water at different temperatures ranging from 60° F. to 100° F. under anaerobic conditions produced a toxin which caused seedlings to wilt and die. No toxin was present in the water from roots incubated at 32° or 40° F. Both oven-dried and field-moist citrus roots when incubated in water under anaerobic conditions produced a toxin that caused citrus seedlings to wilt. Regardless of the initial pH of the water in which the roots were incubated the resultant pH of the root solution was approximately 5.0. This could be attributed to the evolution of CO_2 by microorganisms and the absorption of the CO_2 in the water which became buffered around pH 5.0. It was apparent that there was some buffering in the solution when the pH was being raised or lowered with a strong base or acid. The pH of the root solution after test seedlings had been in it for more than 7 days ranged from one to two units higher than its initial pH. If carbon dioxide were released as the roots respired there should not have been the

change in this direction. It seems likely that the roots have absorbed the substance which caused the solution to be acid and in turn released a basic fraction which influenced the pH of the solution.

It is highly probable that microorganisms enter into the production of the toxin. Attempts were made to isolate them by using different media and to use these to inoculate flasks with sterile roots. Autoclaving roots was the only way to achieve complete sterilization. Even though it was realized that the chemistry of the roots was changed by the process, these flasks were inoculated with bacteria and fungi from the different agar media. All inoculated flasks had prolific growth, but none of the water in the inoculated flasks had enough toxin to cause the seedlings to wilt.

Seedlings wilted in the solution from the partially sterilized roots which had been treated with hypochlorite solutions. This suggests two possibilities. One, the toxin in the root solution which causes seedlings to wilt is produced metabolically as the roots remain in the water under anaerobic conditions. Two, the toxin is a substance produced when the microorganisms attack a root which has been weakened in the presence of a poorly aerated environment. Whichever the reason, the substance is water-soluble. The roots

of seedlings are capable of absorbing it and of translocating it upward. It is unknown whether the wilting is caused by the plugging of the conducting system (48), thus creating a physiological wilt, or whether there is a toxic effect on the protoplasm.

During the anaerobic respiration of the severed roots in the sealed jars full of water a toxic substance could first accumulate in the cells and later, as the cells erupt, the toxic substance could then be absorbed by the water forming a water solution. When citrus seedlings were in an acid, stagnant, waterlogged soil they began to show wilting after one week and later desiccated. After 2 weeks the free water was withdrawn from the soil, filtered, and used to grow seedlings. These test seedlings wilted, not only in the filtered water but in all distilled fractions. As the roots remained in the stagnated waterlogged soil it is probable that the anaerobic respiration of the roots produced a toxic substance which was translocated upward. Also this substance was released to the soil water, but apparently not destroyed by anaerobic microorganisms, and was readily absorbed by the roots of actively growing seedlings. It is believed that there is more than one component in the water which is absorbed by the test seedlings since some seedlings are

bleached, some are bleached and wilted, and some wilted.

Citrus seedlings did not wilt in the free water which was withdrawn from stagnated waterlogged soil with no citrus roots. This seems to indicate that the mere presence of stagnated water in soil does not cause the citrus seedlings to wilt but that a toxic substance is formed when citrus roots are in stagnated water in soil. It has been noted that when citrus seedlings were left in non-aerated water or nutrient solution no wilting occurred. However, when the citrus seedlings were in an aerated nutrient solution for several months and the aeration stopped, the roots on the seedlings soon rotted and the seedlings wilted. When a turgid seedling was placed in this water, it wilted within a week. It has not been determined what quantity of roots is necessary to produce enough toxin to cause a citrus seedling to wilt and die. It is conceivable that the root system on a 6-month or older citrus seedling could produce enough of the toxin under acid stagnant conditions to cause the plant to wilt as was indicated by the seedlings which were flooded in cans.

The water-soluble toxin was more detrimental to the citrus seedlings in an acid medium than in a basic

medium. This was found to be true when the acid root solution was made basic and when seedlings were flooded in a slightly basic soil. Ether extracted the toxin from an acid root solution but not from a basic root solution. No identification has been made; however, it could be an organic ester which is saponified and made somewhat inactive when cations are present and the solution is basic.

The substance was found to be thermostable and distillable. The larger the quantity of roots per unit of water the sooner the plants wilted in both the undistilled fractions and the distilled fraction. The solutions from lateral roots caused the seedlings to bleach nearly white, as did the distilled fractions. The last distilled fraction had a faster and a more complete bleaching effect than the other fractions on the seedlings. The thermostability of the toxin was shown by its presence equally in the distillate from boiling and from vacuum distillation at 45° C. When the root solution was made basic before evaporating under vacuum or at room temperature the evaporation of the toxin was suppressed. The substance might be an ester that does not evaporate from a basic medium but is readily evaporated from an acid medium. When the root solutions were evaporated to dryness at room temperature, both in

evaporating dishes and from the paper, there was a strong offensive odor. This odor could be associated with the toxin since the toxic substance was removed from the solution when the water was evaporated with heat, vacuum and at room temperature.

There was intense fluoresence in all root solutions. With paper chromatography the root solution was separated into five characteristic fluorescent spots. None of these spots when eluted from the paper caused the seedlings to wilt. Fluorimetric analysis was made on the eluted spots and the distilled fractions. There was no correlation between the wilt of seedlings and the fluorescent intensity.

Extracts from soil which had been planted with citrus seedlings (59) and old citrus trees (89) have been shown to reduce growth of citrus seedlings in a well aerated medium. It would seem worthy of further investigations to see if this growth-reducing substance was related to the toxin in the root solution which caused seedlings to wilt. Growth of other plants, for example peach (70) and guayule (8), were reduced by toxic substances which have been extracted from soil which had been previously cropped with the same plant species.

V. DETECTION OF VIABILITY OF CITRUS SEEDLING ROOTS

When citrue roote are damaged in waterlogged eoils the cortex generally eloughe easily. This ie ueually an advanced stage of injury. If a method were devieed whereby root injury could be detected before advanced injury to the top was evident, thie would be useful in determining how long a plant could tolerate water before the root was injured and be a helpful method in determining the rate at which drainage of the soil would be neceeeary to eliminate large amounte of root injury. A method to be useful should be rapid and easy to use under field conditione.

Methods and Results

Rough lemon, Cleopatra mandarin, sour orange and sweet orange seedlinge grown in the greenhouse were used in the investigations. An oxidation-reduction material, 2,3,5-triphenyltetrazolium chloride ealt (TTC) was used as the indicator to test for live tissue. Hot water, propylene oxide, malonic acid eolution and acetic acid were used to kill the roots. Also the roote on citrus eeedlings which had been in flooded eoile, in hydrogen eulfide eolution and in deoxygenated water were teeted for viability with the

tetrazolium salt.

Experiment 1.- Preliminary testing of TTC for staining citrus roots.

Roots from citrus seedlings were placed in 60° C. water for 30 minutes. Following this treatment these roots and untreated roots were placed in a 1 per cent TTC solution overnight. Roots from healthy citrus seedlings were placed in a 0.1 per cent TTC solution for 3 hours under laboratory conditions, in direct sunlight for 10 minutes, and in a 0.5 per cent solution under a light source of 500-foot candles for 1 hour.

All of the fresh roots in the TTC solutions were stained red. The fresh roots in the 1 per cent solution overnight were more intensely red than the roots in the 0.1 per cent solution either under laboratory light or in direct sunlight. The roots in the 0.5 per cent solution were more intensely stained than the roots in the 0.1 per cent solution under the laboratory lights or sunlight. Regardless of the concentration of the TTC solution the longer the fresh roots remained in the solution the redder they became. Only the solution in the direct sunlight turned red. None of the roots treated with hot water stained red.

Experiment 2.- Determining the viability of citrus roots using a TTC solution.

The roots of Rough lemon and Cleopatra mandarin seedlings were immersed in propylene oxide for 15 minutes and for 2 hours, and in a 1 per cent malonic acid solution for 1 hour and for 2 hours. Following the time of immersion in the 2 solutions the roots were rinsed and placed in a 0.5 per cent TTC solution under a light intensity of 500-foot candles for 1 hour. Roots of untreated seedlings were placed in the TTC solution as check plants. After the TTC treatment the seedlings were planted in a sand-peat mixture in the greenhouse and watered.

None of the roots on the seedlings treated with propylene oxide stained red in the TTC solution. The feeder roots after 1 hour in the malonic acid solution stained red in the TTC solution. Only the tap root of the root systems in the malonic acid solution for 2 hours turned red in the TTC solution. The entire root system on the check plants was stained red.

After 24 hours in the sand-peat mixture the leaf veins on the check plants and the plants treated with malonic acid were red and the plants were turgid. The stems of the seedlings that were in the propylene oxide for 2 hours were blanched but the leaves remained turgid. By the third day the Rough lemon seedlings treated with malonic acid were wilted. After one week

only the Cleopatra mandarin seedlings that were treated with only the TTC solution remained turgid.

The roots of Rough lemon seedlings were exposed to propylene oxide vapors for 15, 30, 45 and 60 seconds, 2, 3, 4, 5, 10 and 30 minutes. The root system was suspended in a 500-ml Erlenmeyer flask (with a rubber stopper) in which there was a small amount of propylene oxide. After the roots were in the flask, the flask was rotated gently in order to saturate the air in the flask. The plants were removed and placed in a 0.5 per cent TTC solution. Following the TTC treatment they were placed in beakers of water in the greenhouse. Unexposed seedlings in TTC solution were used as checks.

Only the feeder roots exposed to propylene oxide vapors from 15 seconds through 3 minutes turned red in the TTC solution. The tips on the newly formed roots had turned red on all the roots exposed to propylene oxide vapors from 15 seconds to 5 minutes. The leaf veins on all of these seedlings were red, but the leaves on the seedlings whose root systems were exposed to the vapor for 10 and 30 minutes were curled after 2 days in the water. On the fourth day all seedlings exposed to the propylene oxide vapors for more than 60 seconds were wilted.

The roots of Rough lemon seedlings were subjected

to the following treatments, with 8 seedlings for each treatment:

Treatment 1- 1 per cent malonic acid solution for 1 hour then in the 0.5 per cent TTC solution.

Treatment 2- 1 per cent malonic acid solution for 1 hour.

Treatment 3- 70° C. water for 30 minutes, then in the TTC solution.

Treatment 4- 70° C. water for 30 minutes.

Treatment 5- TTC solution.

Treatment 6- Deionized water at room temperature.

After the various treatments one-half of the seedlings in each treatment were placed in beakers of water and the other half were planted in Lakeland fine sand. After the seedlings were planted the sand was watered but not saturated.

The roots on the seedlings that were first immersed in the malonic acid solution then the TTC solution were as red as the roots on the seedlings that were immersed in only the TTC solution. None of the roots from the hot water treatment stained red in the TTC solution. All seedlings treated with malonic acid and hot water, with or without TTC, and placed in water or in soil were wilted the third day. Two weeks after each

treatment the seedlings in water from the malonic acid treatment were wilted but still green whereas those in the soil were desiccated and brown; but the TTC-treated seedlings and the untreated seedlings in both the water and soil were turgid and green.

Experiment 3.- Effect of deoxygenated water on the viability of roots of sour orange seedlings.

Nitrogen gas was bubbled through deionized water to remove soluble oxygen. There was no measurable oxygen content of the water as determined by the A.O.A.C. (1) method. Sour orange seedlings were suspended in glass bottles with rubber stoppers and the tops sealed with paraffin. There were two series of 5 bottles each connected together. Nitrogen gas was passed through the bottles prior to filling the bottles with the deoxygenated water. Five bottles, not connected, were filled with deionized water and 1 seedling was placed in each bottle. The seedlings in the bottles remained in the greenhouse for 1 month. When the seedlings were removed the roots were placed in a 0.5 per cent TTC solution.

All seedlings were turgid after one month in the water. There were new roots on all of the sour orange seedlings in the deionized water but no new roots on those seedlings in the deoxygenated water. The taproots after 1 hour in the TTC solution turned red on 7 of the

10 seedlings from the deoxygenated water. Over 75 per cent of the feeder roots on these seedlings had begun to slough. The roots on the 3 seedlings which did not stain red were darker and the cortical portion of the roots sloughed rather easily. The taproot and the new roots on each seedling from the deionized water all stained red after 1 hour in the TTC solution.

Experiment 4.- Effect of hydrogen sulfide solution on citrus seedlings.

Generated hydrogen sulfide was bubbled into deionized water. The concentration of dissolved hydrogen sulfide was determined by the A.O.A.C. method. Fourteen bottles of 270-ml capacity were filled with a hydrogen sulfide solution containing 1.07 mg sulfide per ml. One Cleopatra mandarin seedling was placed in each of 6 bottles, 1 Rough lemon seedling in each of 5 bottles, and 2 sour orange seedlings in each of 3 bottles. Each bottle was sealed with a rubber stopper and paraffin which supported the seedlings. After 9 days in the solution the seedlings were removed and the roots placed in a 0.5 per cent TTC solution.

None of the roots turned red after the TTC treatment. All of the seedlings were wilted and the stems were bleached. The roots sloughed easily. Only the leaves on the Rough lemon seedlings had brown edges.

Experiment 5.- Effect of acetic acid on citrus seedlings.

The roots of Rough lemon, Cleopatra mandarin, sour orange and sweet orange seedlings were immersed in approximately 0.2 N and 0.1 N acetic acid for 17 hours at which time the roots were removed from the acetic acid solutions and immersed in a 0.5 per cent TTC solution.

None of the roots turned red in the TTC solution. Only the leaves on the sour orange seedlings were not wilted after 17 hours in the acetic acid. More of the leaves on the wilted seedlings in the 0.2 N acetic acid were brown. After 46 hours in the acetic acid solutions all of the stems and leaves were brown and wilted.

Experiment 6.- Tetrazolium as a test for viable tissue in roots of citrus seedlings that had been in flooded soils.

Sweet orange seedlings that had wilted in flooded soils were removed from the soil, and their roots were rinsed and placed in a 0.5 per cent TTC solution. None of these roots turned red.

Rough lemon, Cleopatra mandarin, sour orange, and sweet orange seedlings were flooded in 46-ounce metal cans filled with Leon topsoil (pH 4.5). Four seedlings of each variety were removed from the cans after 2, 4, 6, 9, 14, 21, and 39 days of continuous flooding. The roots from each seedling were placed in a 0.5 per cent TTC solution and examined for red color after 1 hour

under a 500-foot-candle light. The roots were removed from the TTC solution and each root system was placed in 10 ml of acetone overnight to extract the formazan. The optical density of the acetone-formazan solution for each root system was determined on a Beakman DU Spectrophotometer. The roots were oven-dried and a specific extinction coefficient (K) was calculated for each solution of each root system. The results are presented in Table 14.

The K values for the Rough lemon, sour orange, and Cleopatra mandarin roots were much smaller after 4 days of flooding than after 2 days of flooding. However, there was little change in the K value for the sweet orange roots, which had after 2 days the same K values as the others had reached in 39 days. During the first 3 weeks of flooding the Rough lemon, sour orange and Cleopatra mandarin roots maintained higher K values than the sweet orange roots. However, after 39 days of flooding the average K value for all seedlings ranged from 10-20. The visual observations were closely allied to the calculated K values. The longer the roots remained in the flooded soil the greater number of lower roots sloughed. Adventitious roots grew at the base of the stem just beneath the soil surface.

TABLE 14.- Specific Extinction Coefficients for flooded root systems of citrus seedlings treated with a triphenyltetrazolium chloride solution and the formazan extracted with acetone.

Seedling			Specific Extinction Coefficient (K)*						
			Days following flooding						
			2	4	6	9	14	21	39
RL	1		103	48	44	42	52	25	16
	2		138	26	50	41	49	10	10
	3		137	20	57	55	47	26	16
	4		212	36	56	38	49	33	21
		Av.	148	32	52	44	49	23	16
SO	1		117	35	22	22	35	13	8
	2		100	100	10	27	32	21	11
	3		148	34	21	17	19	36	9
	4		94	46	20	20	28	26	7
		Av.	115	54	18	22	28	24	9
SwO	1		10	18	9	13	9	7	10
	2		20	14	12	22	12	6	12
	3		16	16	9	14	9	7	8
	4		12	13	7	8	14	7	-
		Av.	14	15	9	14	11	7	10
Cleo	1		92	13	37	38	37	17	21
	2		118	16	31	28	33	16	22
	3		110	8	35	34	35	11	17
	4		178	15	19	28	28	15	17
		Av.	125	13	31	32	33	15	19

* Specific Extinction Coefficient (K) = $\frac{\text{optical density}}{\text{dry wt. of roots per 10 ml acetone}}$

Discussion

Before testing citrus roots from flooded soils for viability the roots of citrus seedlings were subjected to environments which would either kill them immediately or gradually. Hot water (60-70° C.), malonic acid solutions, propylene oxide (vapor and liquid), hydrogen sulfide solution and deoxygenated water caused citrus roots to show little or no reaction when immersed in the TTC solution following any of the above treatments. On the other hand, citrus roots on actively growing seedlings which were removed from the soil and washed with running water turned red in the TTC solution. The red color was due to the reduction of the colorless triphenyltetrazolium to the red triphenyl formazan. This reduction is indicative of an actively respiring organ. It has been found (74) that once the formazan is formed in the tissue it is not translocated. The colorless TTC was absorbed by both the injured and non-injured root systems as was evidenced by the red veins of the leaves and the red stems of the seedlings with and without injured root systems.

The malonic acid, propylene oxide and deoxygenated water caused injury to the feeder roots before the tap root. When the roots were placed in the TTC

solution following the various treatments the feeder roots remained colorless while the tap root on each plant showed some degree of red discoloration. Apparently the respiration of the feeder roots was impaired, thereby inhibiting reduction of the TTC upon absorption. There was a direct relation between the length of time the roots were subjected to propylene oxide and the malonic acid solutions and the amount of redness in the root tissue indicative of the reduction of the absorbed TTC solution.

Malonic acid was used because of its known effect on the respiratory cycle. Malonic acid will penetrate tissues of higher plants only from solutions of relatively low pH. This acid is an inhibitor in the respiratory cycle, and competes for the hydrogen ion, thus interrupting the cycle, and causes injury to the tissue. A 1 per cent solution of malonic acid was sufficient to inhibit the respiratory cycle of the citrus seedlings, causing these seedlings kept in water to wilt or to desiccate when planted in soil. The roots in the water were under less tension than those in the soil. Therefore those in the water following the malonic acid treatment remained green, even though wilted, while those in the soil desiccated in the same length of time.

In flooded soils lack of oxygen, the build-up of hydrogen sulfide and of organic acids have been given as reasons for root injury. Citrus seedlings that were in deoxygenated water for 1 month had sloughed feeder roots but the tap roots remained intact and these stained red when placed in the TTC solution. Citrus seedlings that were in deionized water for 1 month did not have sloughed feeder roots but new roots had formed, all of which stained red in the TTC solution. Both hydrogen sulfide and acetic acid were toxic to the roots thus causing the seedlings to wilt and desiccate. The roots sloughed, the stems bleached and there was no reaction when placed in the TTC solution. Even though all of these conditions caused injury to roots of citrus seedlings it is doubtful that one cause can be singled out to be the reason for the root injury and subsequent injury to the aerial portion.

The viability of the roots of citrus seedlings which have been subjected to a flooded soil was impaired to a greater extent after 4 days than after 2 days. Even though the viability of the roots as determined by the TTC solution test decreased during the 39 days of flooding, there was no evidence of injury to the tops of the seedlings. The roots on seedlings which had wilted in a flooded soil showed no viability with the TTC

solution. Viability of roots can be impaired without causing death of the plant. However, when the roots show no viability the tops of the seedlings are wilted even though the roots are bathing in water. When a plant is physiologically wilted the viability of the roots has been impaired and there will be no reaction with the absorbed TTC. The precise causes of this physiological wilt are still unknown; however, it is doubtful that free water in soil or lack of oxygen can be the main reasons. Many substances can inhibit the viability of roots. Whether the inhibition is due to the interference with the respiratory cycle, the plugging of the conducting tissue, or possibly to the combination of these, still has to be determined. If TTC is to be used as an indicator for tissue viability the TTC must be reduced. If production of reducing substances is inhibited completely in the plant tissue the absorbed TTC will not be reduced thus causing no red coloration of the tissue. Whenever the citrus roots showed no coloration the plants wilted within a few days after the treatment which killed the tissue.

VI. SUMMARY

1. Seedlings of Rough lemon, sour orange, sweet orange and Cleopatra mandarin, which are commonly used for rootstocks, were flooded under greenhouse conditions while growing in Leon topsoil and Leon subsoil (the leached layer).

2. None of the seedlings flooded in January showed any water injury during a twelve-month period of flooding.

3. All seedlings were injured sooner when flooded in June, July and August than when flooded in April.

4. The seedlings in the subsoil were injured sooner and more severely after they were flooded than were the seedlings in the topsoil.

5. The injury index rating for Cleopatra mandarin, sweet orange, sour orange, Rough lemon, Rusk citrange, Troyer citrange and Carrizo citrange seedlings in the acid subsoil was higher after each week of flooding than the injury index rating of the seedlings in the subsoil to which dolomitic limestone had been added. In the unlimed soil the order of decreasing tolerance to the free water was Troyer citrange, Carrizo citrange, sour orange, Rough lemon, sweet orange, Cleopatra mandarin and Rusk citrange.

In the limsd soil there was no difference in the injury rating among the Troyer oitrange, Carrizo citrange, sour orange and Rough lemon seedlings (which ware the most tolerant group of the seedlings); the sweet orange, Cleopatra mandarin and Rusk oitrange were equally the least tolerant. New root growth was best on the Rough lemon, Troyer citrange and Carrizo oitrange seedlings in the limed flooded soil while in the acid flooded soil the Troyer citrange and sour orange seedlings had the best new root growth though it was very much infsrior to that of the seedlings in the limed soil.

6. Triphenyltetrazolium chloride solution was used to differentiats between viable citrus roots and dead citrus roots. Viable roots stained red while dead roots did not.

7. Toxic substances, wilt-induoing to citrus seedlings, were formed in the water in which citrus roots were incubated under anaerobio conditions at temperatures from 60° F. to 100° F., but not at 32° F. or at 40° F.

8. Healthy citrus seedlings wilted in the watsr which was withdrawn from flooded Leon soil in whioh oitrus seedlings had wilted two weeks after the cans were flooded. Citrus sesdlings in the distilled fractions of the soil water extract were wilted, bleached, or

both. No seedlings wilted in the water withdrawn from flooded Leon soil without citrus roots nor in its distilled fractions.

9. The water-soluble toxic substances were more detrimental to citrus seedlings in an acid medium than in a basic medium.

10. Ethyl ether removed the toxic substances from an acid root solution but not from a basic root solution.

11. The toxic substances were thermostable and distillable.

12. Both the root solutions and the flooded soil extracts where citrus roots had been present had intense fluorescence. There was no apparent correlation between the fluorescence and the wilting of citrus seedlings in the distilled fractions of root solutions or soil extracts.

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BIOGRAPHICAL SKETCH

Rubert W. Prevatt was born May 15, 1925, at Seville, Volusia County, Florida. He attended the public schools of that county and graduated from Deland High School in 1943. At the University of Florida he majored in soil chemistry and received the degree of Bachelor of Science in Agriculture in June, 1948. During his undergraduate study he was honored by election to membership in Alpha Zeta fraternity. He served as laboratory assistant in the Department of Soils, Florida Agriculture Experiment Station, while he conducted his graduate studies for the degree of Master of Science in Agriculture which he received in June, 1951.

Following his graduation he was soil chemist and assistant to the grove superintendent for the Dr. P. Phillips Company, Orlando, Florida. He entered Cornell University in September, 1954, and transferred to the University of Florida in September, 1956, where he pursued his studies for a degree of Doctor of Philosophy.

This dissertation was prepared under the direction of the chairman of the candidate's supervisory committee and has been approved by all members of that committee. It was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was approved as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June 8, 1959.

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